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PROTEIN HORMONES OF THE PITUITARY BODY

BY

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H. L. FEVOLD, GEORGE W. IRVING, JR., C. N. H. LONG,
THEODORE SHEDLOVSKY, AND ABRAHAM WHITE

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INTRODUCTION TO THE CONFERENCE ON PROTEIN HORMONES OF THE PITUITARY BODY

By H. B. van Dyke

From the Division of Pharmacology, The Squibb Institute for Medical Research, New Brunswick, N. J.

This gathering of physicists, chemists and biologists has a general or specialized common interest in those fragile giant molecules, the proteins. The curiosities, the languages and the tools of the investigators, however, are otherwise so conspicuously different as to make even Dr. Shedlovsky despair of any attempt to apply "criteria of purity" to the whole group. Perhaps the most important benefit which can come from this conference is the opportunity of learning with what confidence we can speak of the isolation and the physical or chemical characterization of those pituitary hormones which are believed to be proteins. If that purpose is to be accomplished, discussion must be free and frank. In contrast with the limitations imposed at the usual large scientific gathering today, it is fortunately possible not to have to demand serious curtailment of pertinent discussion during the two days at our disposal. The biological aspects of discussion will have to be limited, however, to those phases which must be understood and defined by the investigator interested in proteins extracted from the pituitary. To the profit we can all expect from a complete exchange of data and ideas can be added the pleasure described by Nietzche's epigram, "It is certainly not the least charm of a theory that it is refutable." I hasten to add, however, that conferences like this unfortunately are now scientific luxuries whose number will necessarily be increasingly restricted.

Those members of the conference who are not biologists should be acquainted with the general topography of that complex domain, the biology of the pituitary gland. In an effort to serve only briefly as a guide, I shall have to be dogmatic and speak simply of those confusing phenomena in which biological specialists lose themselves and their readers.

The endocrine or ductless glands are dominated by the pituitary body, distinguished from all the others by its structural intricacy of four distinct parts. Nothing is known of the function of one division, the *pars tuberalis*, which is found as a collar of tissue about the stalk of the pituitary of mammals. This stalk, by which the gland is attached to the base of the brain, is a further distinction of the pituitary in the glandular hierarchy and permits nervous centers, especially of the primitive part

of the brain, to dispatch impulses to a second lobe lying to the back, the neural lobe. A third division, the intermediate lobe, lies between the neural lobe and the fourth part, the anterior pituitary, which, although last on our list, is the largest and most important part.

The physiological dominance of the pituitary among glands of internal secretion depends exclusively upon this largest part, the glandular or anterior lobe. This fact has been demonstrated by all the traditional methods of the endocrinologist. If this lobe has been removed, no other important gland of internal secretion can function to the proper benefit of the whole organism. For example, the thyroid gland, which regulates the pace of heat production in the body, atrophies and secretes only seriously inadequate quantities of its hormone so that the metabolic rate falls and the body temperature is significantly reduced. On the other hand, a suitable anterior pituitary extract will cause the opposite effects if injected: the thyroid hypertrophies; its cells secrete at an abnormally rapid rate; the metabolic rate rises; other signs also resembling overdosage of thyroid extract appear. A similar picture differing in details could be described with respect to the adrenal glands, the ovaries and the testes. The substance or substances regulating growth or metabolism (or both) often act directly, but probably depend upon the presence of one or more endocrine glands, such as the thyroid or adrenals, to exert their full effects. Therefore, the anterior lobe of the pituitary regulates growth, reproduction and metabolism (utilization of food) either directly by acting on non-glandular tissues or indirectly by maintaining or stimulating the important endocrine glands.

For a number of reasons the importance of the anterior pituitary regulation of the gonads (ovaries and testes) has received special attention reflected by intricate hypotheses regarding the controlling mechanisms. The diagram of FIGURE 1 may assist those not familiar with pituitary physiology. At present, the testes appear to be controlled by only two pituitary secretions. Follicle-stimulating hormone maintains the tissue (tubules) in which the male germ cells divide and mature to become spermatozoa, whereas luteinizing hormone stimulates the interstitial cells which secrete the male hormone or androgen necessary for secondary sexual characteristics. Three anterior pituitary secretions appear to direct and operate the complex functions of the ovary. The follicle-stimulating hormone is necessary for complete follicular development culminating in the release of the ovum (ovulation). The remaining cells of the follicle are then transformed into lutein cells (*corpus luteum*) owing to the action of the luteinizing hormone. The latter acts only as a capitalist; it furnishes one of the essential means of erecting the new struc-

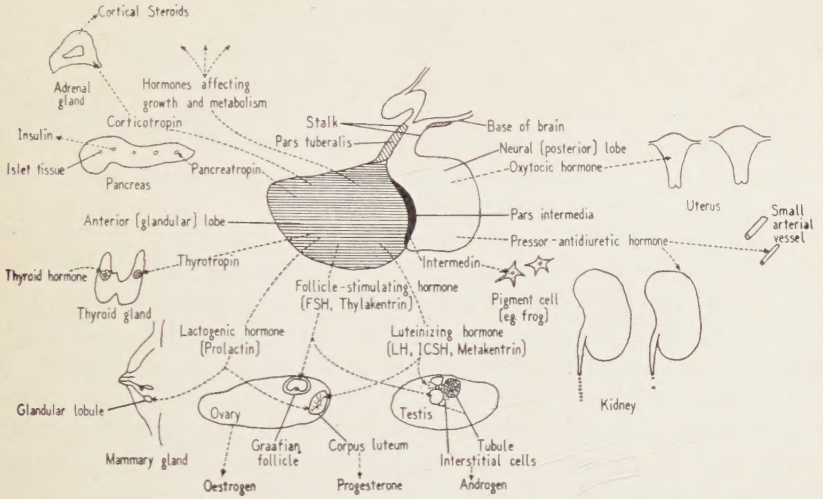

FIGURE 1.—Diagram of pituitary hormone physiology.

ture but contributes nothing to the operation of this luteal body which immediately languishes and has no useful function with respect to pregnancy unless a third pituitary hormone, the lactogenic hormone, is secreted to furnish the managerial or operative stimulus. Thus, lactogenic hormone, so named because its first demonstrated effect was upon the secretion of milk, makes possible the secretion of progesterone which, acting on the prepared uterine mucosa, permits implantation and growth of the fertilized ovum. Ovarian secretion of estrogen and ovulation appear not to occur unless both follicle-stimulating and luteinizing hormones are acting either simultaneously or in close sequence.

Lactogenic hormone likewise supplies the operative stimulus for the secretion of milk but cannot function unless the breasts are prepared for lactation.

Other anterior pituitary hormones are shown in the diagram as stimulating general body growth, affecting metabolism, stimulating the thyroid, the adrenal cortex and the islet tissue of the pancreas. The least striking of these actions is the stimulation of pancreatic islet tissue. The effects on general body growth and particularly those on closely related metabolic processes are ill understood, and, as Dr. Long emphasizes, will not be better comprehended until approximately pure hormones are available for study. Thyroid and adrenal stimulation are peculiarly important because thyroid hormone and the adrenal cortical steroids are so essential for the normal utilization of food and minerals.

Unfortunately, no qualified investigator was able to present a discussion of intermedin, the melanosome-dispersing or chromatophorotropic hormone of the *pars intermedia*. Probably this hormone is a polypeptide. So far as we know the survival of cells secreting intermedin in the mammalian pituitary represents an atavic heirloom since intermedin is principally of interest as a hormone dispersing pigment granules in cells of certain fishes, amphibia and reptiles.

The neural lobe as its name suggests is the only division of the pituitary derived from nervous tissue to which it bears considerable resemblance. The anatomical and secretory integrity of this lobe depends upon an intact connection with the base of the brain by means of the stalk of the pituitary. Extremely active substances causing the contraction of blood vessels, a lessening of the rate of urine formation together with a change in the minerals excreted in the urine, and a contraction of the uterus, can be extracted from neural lobe tissue. The physiological importance of this lobe is most evident with respect to the metabolism of water so far as this is regulated by the kidneys.

The biologist must accept a grave responsibility in performing accurate qualitative and quantitative assays. Here lie fruits of great dissension to which another conference like this could devote its whole proceedings. So far as the general field is concerned, the accuracy, speed and convenience of assay of neural lobe principles have not been excelled. Methods of assaying anterior pituitary hormones are so diverse and are commonly so lacking in accuracy as to represent the biologist's great contribution to much of the existing confusion which can be exorcised only by tolerance, thought, labor and yet more labor. Finally it is well to remember that all qualitative assays (attribution of specific biological effects to extracts) require that the animals used for assay be hypophysectomized. Also, it is increasingly evident that hypophysectomized animals should be chosen for many of the attempts to perform quantitative assays.

In the beginning, I warned you that I should be dogmatic. Although I believe that I have justified that warning, I have frequently been forced to qualify my remarks. Since our knowledge is so imperfect, it should be evident that, so far as events allow, physicists, chemists and biologists must continue to ratify and utilize an alliance which permits all tools of research to be used in the isolation of pure pituitary hormones. Only by this means can we hope ultimately to understand how this extraordinarily complex and dominant gland may ensure health or contribute to disease.

CRITERIA OF PURITY OF PROTEINS

By Theodore Shedlovsky

From the Laboratories of The Rockefeller Institute for Medical Research, New York

INTRODUCTION

A pure chemical substance may be defined in a simple, unambiguous manner as *a quantity of matter consisting of a single molecular species.* However, this definition excludes allotropic, polymeric, isotopic and other "mixtures" which, for many purposes, can usefully be considered to be pure substances. For example, what is called pure water contains molecules composed of various isotopes of hydrogen and oxygen, probably some polymerized molecules, hydrogen ions, deuterium ions and hydroxyl ions of several isotopic compositions.

The range of the usual molecular weights for proteins is from 20,000 to 200,000 and higher. This corresponds to large numbers of atoms in each molecule. Are we to suppose that these atoms have the same spatial arrangement in all the molecules of a pure protein? Probably not, although we have no experimental way of answering the question. But, since we are not interested in philosophical wrangling, we shall confine our discussion to operational considerations of chemical purity. Moreover, in this conference on protein hormones we are chiefly interested in knowing whether a particular biological activity is due to most of the substance in a certain preparation or to an impurity in it. We shall, therefore, lay our emphasis on impurities rather than "purity."

The usual operational criteria for the purity of inorganic and organic materials, which are not megamolecular as are proteins, are constancy of density, refractive index, optical rotation, melting point, boiling point, dielectric constant, electrical conductance, solubility, analytical data, etc., after redistillation, recrystallization, or preparation by different methods. Unfortunately, most of these operations are not available for proteins. These are very labile substances, and the procedures which can be used without fear of profoundly altering them are indeed limited. Also, analytical data on proteins are of relatively little use in most cases for establishing purity, and laboratory synthesis has as yet not been possible.

Among the various physicochemical procedures which are applicable to the study of proteins there are a few which provide the most satisfactory criteria we have for estimating the degree of purity. These are electrophoretic analysis, observations in the analytical ultracentrifuge,

and the determination of solubility curves in suitable solvents. Proteins form salts with both acids and bases, and, except at the isoelectric point, appear as ions with a net electric charge. Their electrical mobilities depend largely on the pH and on the salt composition of the solution at a fixed temperature. Two different proteins may have identical mobilities in a given solvent, but the probability of the mobilities remaining similar at other values of pH is much smaller. The ultracentrifuge determines sedimentation constants, which depend on the size and shape of the molecules. Here again, two different proteins may happen to have similar sedimentation constants under certain conditions. Determinations of solubility curves involves analogous considerations. However, the likelihood of two different substances behaving alike in all three respects, that is, electrophoresis, sedimentation and solubility, can probably be ruled out in the present state of our knowledge.

TESTS FOR PURITY OF PROTEINS

Before entering on a discussion of various procedures which may be of use in assessing the degree of purity of a certain protein preparation it may be well to classify the following cases: (1) The pure form is available. (2) The pure form is not yet known. (3) The nature of a suspected impurity is known. In the first case the task is simplified by the fact that all the properties of the preparation to be tested can be compared quantitatively and directly with the pure substance. In the second case one is embarking on an uncharted course and the task is correspondingly more difficult. In the third case sensitive tests for the suspected impurity may already be available which can greatly simplify the work required.

In the sections below, various procedures pertinent to our problem will be discussed. The reader is also referred to articles by E. J. Cohn,[1] (physical-chemical characteristics of protein molecules), and by N. W. Pirie,[2] (criteria of purity of large molecules) which deal with the question.

CHEMICAL ANALYSIS, TITRATION CURVES, ETC.

Different proteins are so nearly alike in elementary composition of carbon, oxygen, hydrogen, and nitrogen that assay for these elements cannot tell us whether we are dealing with one protein or several. The determination of sulfur, phosphorus, metals, or of specific groups such as amino acids and carbohydrates, are much more useful, since in many cases they tend to show appreciable differences from protein to protein. Here, much depends on the magnitude of the differences and the accuracy

[1] **Cohn, E. J.** Chem. Rev. **24**: 203. 1939.
[2] **Pirie, N. W.** Biol. Rev. Cambridge Philos. Soc. **15**: 377. 1940.

of the analytical methods in relation to the proportions of the proteins in a given mixture. The usual analytical methods are sufficiently familiar as are the procedures for estimating specific groups. The estimation of amino acids in a protein from titration curves was discussed by Cannan[3] at a recent conference of the Academy. Analytical results can give valuable information of a negative nature, *i.e.*, yield values which fail to be reproducible from preparation to preparation of what was thought to be one substance, and they can also be helpful in the estimation of molecular weight. But if the analytical figures are reproducible, that fact alone provides a poor criterion for purity.

CRYSTALLINITY

Crystallinity of a protein had been considered an indication of purity, but it is now well recognized that it is an unsatisfactory criterion. To be sure, a homogeneous crystalline preparation assures the absence of substantial amounts of amorphous matter. Also, it is well known that certain proteins which appear to be quite similar in many respects, such as hemoglobins from several species, have different crystal forms. However, different crystal forms may be obtained from some single substances, and the crystallinity criterion suffers from other serious limitations.

Crystals which may appear to be homogeneous under a microscope may consist of mixed crystals or solid solutions. Isomorphism is far from uncommon with proteins. There is always the possibility of occluded matter being present which cannot be removed readily even after many recrystallizations. Preparations may seem to be superficially crystalline but actually may not be so. X-ray analysis can decide this question. It is, in fact, the only reliable means of ascertaining the degree of internal atomic regularity. Also, electron microscopy, which is becoming more common, may supply interesting information about structure.

ELECTROPHORESIS

Electrophoresis is concerned with the motion of charged particles by the action of an electric field. With the moving boundary (Tiselius) method which has become such a powerful tool in the study of proteins and other large molecules, it is possible to obtain very useful information as to the purity of such materials. It is also possible to effect the separation of components from a mixture, at least in sufficient amounts for many further biological studies.

In principle the method consists in sharply stratifying the solution to be studied below the solvent itself in a U-tube. With proteins, whose

[3] **Cannan, R. K., Kibrick, A., & Palmer, A. H.** Ann. N. Y. Acad. Sci. **41**: 243. 1941.

mobilities are sensitive to pH because of their amphoteric nature, buffer solutions against which the preparations are dialyzed serve as solvent. On applying an electric potential all the ions in the tube will move in the field with the result that after a time there will appear boundaries corresponding to the number of components, in addition to those in the solvent, having different mobilities. Also, there will be present the so-called δ and ϵ boundaries which in general move but little if at all. These do not correspond to any components but to buffer salt and total protein gradients left behind in the rising and descending limbs of the tube after the components have moved away from the original boundaries.

The technique which was originally described by Tiselius[4] is now widely used with the improvements of Longworth,[5] who developed the automatic schlieren scanning method, and of Philpot[6] and Svensson,[7] who applied the cylindrical lens and inclined slit method for observing and photographing the boundaries. Thus, electrophoretic patterns are obtained from which the concentrations of the components* having different mobilities can be estimated, and the mobilities can be computed from the volume swept through by the boundaries, the field strength, and the time during which the current had passed.

Electrophoretic patterns obtained with the Longsworth[5] method are shown in FIGURES 1, 3 and 4. The patterns of the various fractions arising in the preparation of crystalline ovalbumin at pH 3.92 are shown in FIGURE 1, taken from the classical paper of Longsworth, Cannan, and MacInnes.[8] The arrows pointing to the left indicate the rising boundaries and the arrows pointing to the right indicate the corresponding descending boundaries. The δ and ϵ peaks correspond to the boundaries arising from salt and total protein gradients, while the others correspond to various components. The areas under the peaks depend on the component concentrations, while the displacements from the tails of the arrows indicate the relative mobilities. A refers to ovalbumin, G_1, G_2 and G_3 to globulins, C to conalbumin and O to ovomucoid. It is evident that the first albumin fraction (b) as well as the filtrate, and the globulin fraction (e) are quite impure, containing components recognized in the complete egg white pattern (a). Repeated crystallization (d), (e), (f) results in a product which is quite homogeneous electrically at pH 3.92. But determinations at other values of pH showed that ovalbumin,

* The optical methods indicate the refractive index gradients in the boundaries. The integrated refractive index gradients correspond to the integrated density gradients which in turn are proportional to the concentrations of the components to which the boundaries owe their origin.

[4] **Tiselius, A.** Nova Acta Soc. Sci. Upsala. IV **7** (4). 1930.
[5] **Longsworth, L. G.** Jour. Am. Chem. Soc. **61**: 529. 1939.
[6] **Philpot, J. S. L.** Nature **141**: 283. 1938.
[7] **Svensson, H.** Kolloid-Z. **87**: 181. 1939; **90**: 141. 1940.
[8] **Longsworth, L. G., Cannan, R. K., & MacInnes, D. A.** Jour. Am. Chem. Soc. **62**: 2580. 1940.

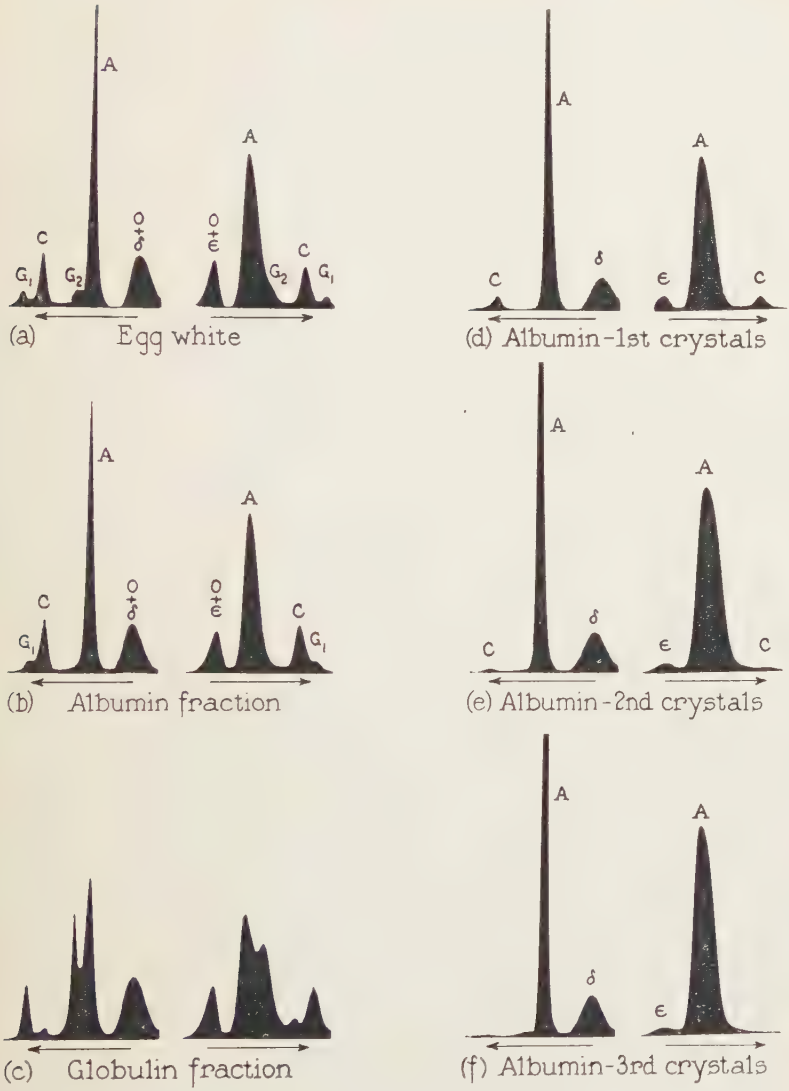

FIGURE 1. Electrophoretic patterns of egg white and its various fractions at pH 3.92 [from Longsworth, Cannan and MacInnes[8]].

A, and conalbumin, *C*, are complex. The mobility curves, as a function of pH, for the components of egg white are shown in FIGURE 2.

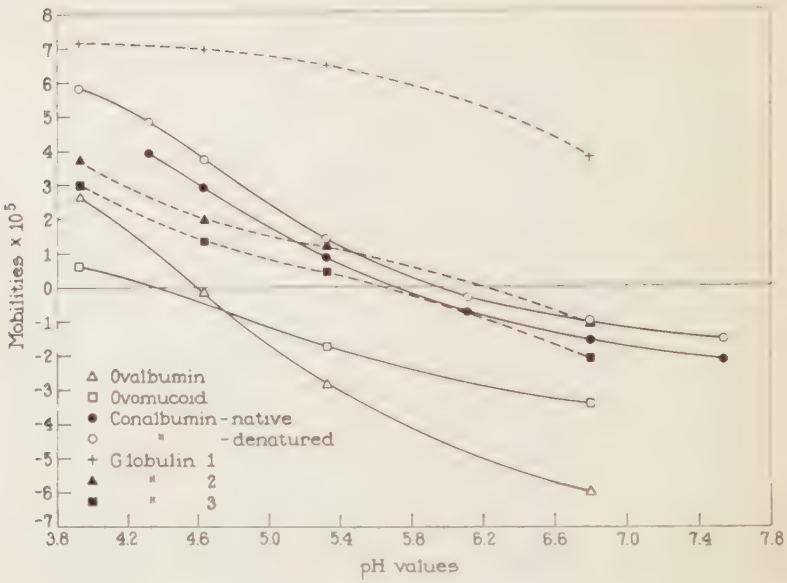

FIGURE 2. Mobility pH-curves for the components of egg white [from Longsworth, Cannan and MacInnes[8]].

The reason for the absence of two conalbumins in FIGURE 1 is due to the fact that a transition takes place between the two forms which is a function of pH. Below pH 4 only one form predominates. The isoelectric points are at the values of pH corresponding to zero mobility.

It is evident that electrophoretic analysis must be carried out at different values of pH to provide as much information as possible regarding purity. Mobility-pH curves in conjunction with relative concentration determinations obtained from the patterns supply the maximum information. It should be noted here that variation of salt concentration (ionic strength) tends to displace the mobility curves. A tenfold change in ionic strength tends to alter the mobility of proteins by an amount corresponding roughly to the effect of one pH unit.[9]

In FIGURE 3(a) a pattern of the rising boundary from a nucleoprotein preparation is shown. The small peaks correspond to soluble impurities, and the opaque region to impurity in the form of a small quantity of suspended matter. With sufficient care the electrophoretic method is capable of detecting a few tenths of a per cent of an impurity which has

[9]**Davis, B. D., & Cohn, E. J.** Jour. Am. Chem. Soc. **61**: 2092. 1939.

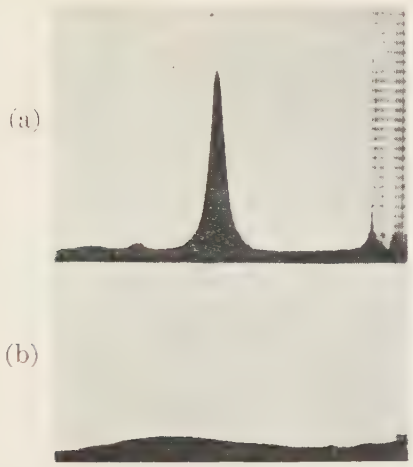

FIGURE 3. (a) Electrophoretic pattern of a nucleoprotein preparation. Rising boundary. (b) Electrophoretic pattern of a protein which had been badly degraded. Rising boundary.

a mobility different from the major substance. FIGURE 4 shows a pattern obtained with the cylindrical lens method[6,7] from a protein preparation which evidently contains at least three components.

Occasionally, one observes considerable electrical heterogeneity of a different sort. The pattern shown in FIGURE 3(b) was obtained from a preparation of protein which had been badly maltreated. It is evident that no sharp boundary exists. The material apparently had been broken up into fragments consisting of particles having a very wide range of mobility.

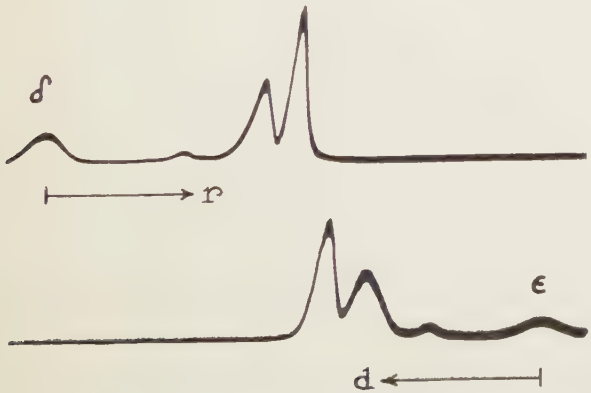

FIGURE 4. Electrophoretic pattern obtained with cylindrical lens of a protein preparation containing three components. Rising boundary (upper curve), descending boundary (lower curve).

Electrophoresis has been very valuable in supplying an excellent criterion of purity for proteins, although it, alone, is not sufficient, since it tells only whether the material is electrically homogeneous or not. As an example of its use in purification, I cite the work of Seibert[10] who used electrophoresis successfully both as a preparative method and as a guiding assay for finding suitable precipitating conditions to purify tuberculin protein contaminated with nucleic acid and polysaccharide. Quoting from her summary: "Nucleic acid and polysaccharide could not be removed quantitatively from the protein by isoelectric precipitation, by electrodialysis, by dialysis at pH 2.1, by repeated precipitation for half saturated ammonium sulfate at the isoelectric point, or by repeated precipitation with 2 or 10% trichloracetic acid. Electrophoretic mobility curves revealed the fact that on the acid side of pH 5 the nucleic acid and the protein migrated as one component, whereas at less acid reactions the two travelled with very different mobilities. The polysaccharide was immobile at all pH values. Therefore by means of repeated electrophoresis at pH 7.3 it was possible to remove both nucleic acid and polysaccharide from the protein with no loss of potency. It was, furthermore, possible to remove both of these impurities from the protein by repeated precipitation on the alkaline side of pH 5, *e.g.*, by half saturated ammonium sulfate at pH 7, with no loss of potency." A detailed electrophoretic study of complex formation between yeast nucleic acid and ovalbumin with a theoretical analysis of the results was reported recently in an important paper by Longsworth and MacInnes.[11]

CENTRIFUGAL SEDIMENTATION

The ultracentrifuge first developed by Svedberg[12] provides a very valuable method for determining the molecular weights of large molecules. Sedimentation is achieved by the use of high speeds which result in sufficiently high centrifugal fields. In most work with proteins, fields of about 200,000 times gravity are produced in rotors spinning at about 60,000 r.p.m. I shall not discuss any of the experimental details beyond stating that the optical systems usually employed at present for observing the sedimentation boundaries resemble those used in electrophoretic work. A detailed treatise on the technique appears in Svedberg and Pedersen's book,[12] and an interesting monograph on the subject has been published recently by the Academy.[13]

[10] **Seibert, F. B.** Jour. Biol. Chem. **133**: 593. 1940.
[11] **Longsworth, L. G., & MacInnes, D. A.** Jour. Gen. Physiol. **25**: 507. 1941–42.
[12] **Svedberg, T., & Pedersen, K. O.** "The Ultracentrifuge." Clarendon Press. Oxford. 1940.
[13] "The Ultracentrifuge." Ann. N. Y. Acad. Sci. **43**: 173–252. 1942.

The sedimentation velocity of particles depends on their density, size, and shape in accordance with the following equations:

$$s = \frac{D(1 - \bar{v}\rho)M}{RT}, \tag{1}$$

$$D(f/f_0) = \frac{RT}{6\pi N\eta}\left(\frac{4\pi N}{3vM}\right)^{1/3}, \tag{2}$$

in which the symbols have the following significance:

M—molecular weight
s—sedimentation constant
D—diffusion constant
T—absolute temperature
R—gas constant
N—Avogadro's number
$\bar{v}$—partial specific volume
ρ—density of the solvent
η—viscosity
f f_0—the shape factor, which is unity for spherical molecules.

If the sedimenting molecules are spherical, the molecular weight can be obtained from the centrifugal data alone, by equation (1), since f/f_0 is unity and D is given by equation (2). Otherwise D must be determined. For obtaining molecular weights it is best to work at a pH near the isoelectric point of the protein. Measurements on diffusion make possible the calculation of the shape factor, f/f_0, from equation (2). Or, conversely, it is possible to determine the degree of asymmetry in the molecular shape if the molecular weight is known. However, if the shape is very elongated, the sedimentation velocity will not be very sensitive to length.

The limitations of the ultracentrifuge as an instrument for providing a criterion of purity are that similar sedimentation constants only indicate similar molecular weights of molecules having the same shape or of compensating values of M and f/f_0. Also, there is a certain dependence on concentration, the sedimentation generally being faster in more dilute solutions. This fact accounts, at times, for spurious sharpness of boundaries; a small amount of slightly smaller material, being in a region of low concentration, will tend to catch up to the leading boundary, thus disturbing the resolving power. However, under favorable conditions, it is possible to detect impurities having sufficiently different sedimentation constants from the major component, if they comprise 2 or 3 per cent of the material.

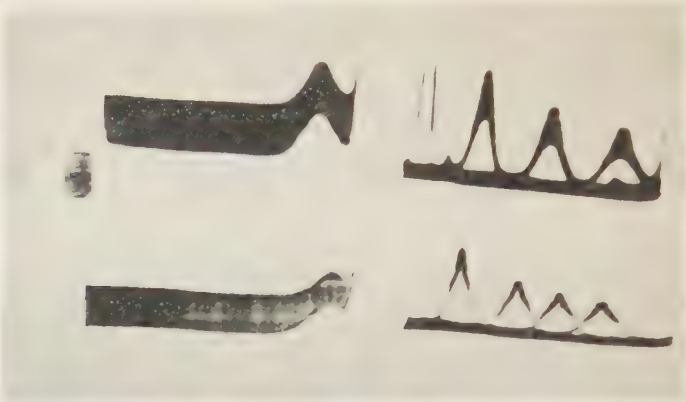

FIGURE 5. Sedimentation patterns. Metakentrin preparations, (a) and (b); Horse globulin preparations, (c) and (d) [Rothen].

The patterns shown in FIGURE 5 were supplied by Dr. A. Rothen. The two on the right are from preparation of hog metakentrin, photographic exposures having been made at intervals as the sedimentation proceeded. A small quantity of a more rapid component (two small peaks) will be noted in the upper patterns, (a). This is absent from another preparation shown in the patterns directly below, (b). On the left of FIGURE 5, centrifuge patterns, (c) and (d) (single exposures), or horse globulin preparations are shown.

As an example of nonhomogeneous material, patterns obtained from urea-denatured ovalbumin (Rothen) are shown in FIGURE 6, which contains several exposures taken at intervals of time during the sedimentation. It should be pointed out, however, that materials which lack homogeneity in the centrifuge need not necessarily also do so in electro-

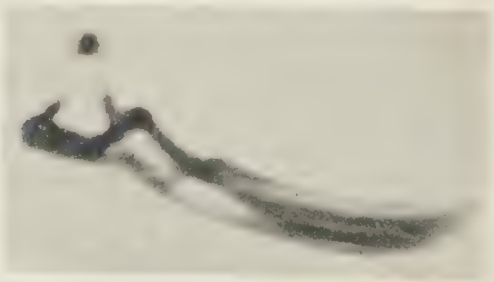

FIGURE 6. Sedimentation patterns of nonhomogeneous material. Urea denatured ovalbumin [Rothen].

phoresis (since mobility depends primarily on charge distribution and not on size and mass), and vice versa.

SOLUBILITY

Theoretically, solubility provides one of the best criteria for the purity of a substance, since it is based on sound thermodynamic principles. It involves the measurement of a partition equilibrium of a substance distributed between two phases. In the case of protein solubility the distribution is between the solid itself (the solid phase) and a solution of it, usually in an aqueous buffered salt medium (the liquid phase). The basic theory which gives quantitative expression for such systems is summarized in the Phase Rule of Willard Gibbs. At equilibrium, the chemical potential of any neutral species is the same in all phases. The chemical potential of a substance in any phase depends on its mol fraction in that phase and on its corresponding activity coefficient which is a measure of deviation from Raoult's law, or "ideal" behavior. A substance will continue to dissolve in a solvent until its chemical potential in the solution builds up to just the value it has in the solid, provided there is sufficient solid available. If the solid is homogeneous (one solid phase) but contains the substance in impure form (solid solution), the chemical potential of the substance is less than it would have been in the pure state because its mol fraction is unity when pure and less than unity when the solid phase contains other components. At equilibrium, the composition of the solid solution is no longer what it was originally, unless the relative solubilities of the components happen to be of just the right ratio. If this should happen to be so, changing the solvent will greatly minimize the likelihood of such compensation. Therefore, it is desirable to make solubility determinations in more than one solvent to derive all the advantages of the solubility method for establishing purity. The addition of more of the original material (solid solution) will again disturb the composition of the solid phase so that, in general, more material will be dissolved. Sorensen,[14] who was the first to apply the solubility method in studying proteins, found that proteins which had been considered pure were actually not so since their solubility increased with the quantity of solid used in the determinations with a given volume of solvent. The method, which has been developed admirably by Northrop and his collaborators[15,16] depends on the determination of the quantity of protein appearing in a constant volume of buffered solvent, at constant temperature and pressure, as the total quantity of

[14] **Sorensen, S. P. L.** Compt-rend. trav. Lab. Carlsberg **15**: 1. 1925; **18**: 1. 1930.
[15] **Northrop, J. H., & Kunitz, M.** Jour. Gen. Physiol. **13**: 781. 1929–30.
[16] **Kunitz, M., & Northrop, J. H.** Cold Spring Harbor Symposia **6**: 325. 1938.

protein is increased. It is best to use solvents which dissolve but little of the material. The concentration of protein, which is proportional to its mol fraction in dilute solutions, is usually determined through nitrogen analyses. The accuracy of the analytical method is one of the important limitations in the technique.

On preparing a plot of total protein against protein in solution, one should obtain, for a pure substance, points which fall on two straight lines. As long as all the material added goes into solution, the points should naturally fall on a straight line with unit slope. When saturation has been achieved no more material should dissolve on further addition of substance, and the subsequent points should therefore fall on a horizontal line, producing a sharp break with the line of unit slope. It is important to get as many points as possible in the vicinity of the break to make sure of its reality.

If the preparation consists of several substances present as a physical mixture, but not in solid solution, we are dealing with several solid phases. Here we will observe several breaks in the plot before it becomes horizontal. Such a plot, for three pure solid phases, is shown in FIGURE 7, taken from Kunitz and Northrop's paper.[7] At point *B* the solution is saturated with substance 1, at point *C* it is also saturated with substance 2, and at *D* it has become saturated with all three. The plot must be horizontal beyond *D* and must have unit slope from *A* to *B*. On extrapolating the lines *BC*, *CD*, and *DE* to the ordinate axis, the intercepts show the solubilities *S*1, *S*1 + *S*2, and *S*1 + *S*2 + *S*3, respectively. In FIGURE 8, also from Kunitz and Northrop,[7] solubility curves for α and γ chymotrypsin mixtures, which do not form solid solutions, are shown.

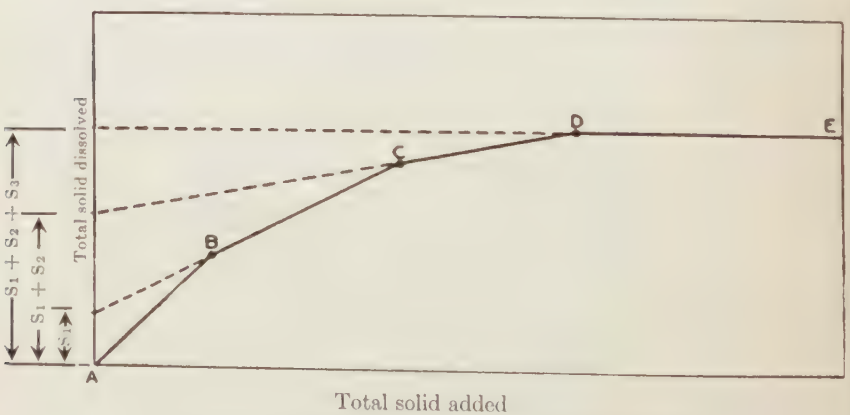

FIGURE 7. Theoretical solubility curve for three pure solid phases.

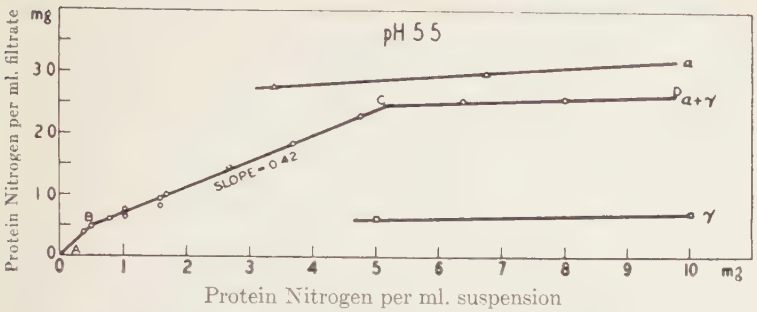

FIGURE 8. Solubility of artificial mixture of crystals of α and γ chymotrypsin (40 per cent α + 60 per cent γ) in 0.4 saturated ammonium sulfate pH 5.5 at 10° C. Slope BC, measured = 0.42, calculated = 0.43. [Kunitz and Northrop[16].]

The uppermost curve, α, and the lowest curve, γ, indicate the solubilities of α and γ chymotrypsin by themselves, from which the slope BC in the middle curve can be obtained if one knows the proportions of α and γ in the mixture, or vice versa. It will be noted that the α chymotrypsin curve slopes gently instead of being horizontal. This indicates a small amount of impurity present in solid solution.

For solid solutions, one does not obtain linear plots with breaks, but curves which show early deviations below the initial unit slope, and which continue to rise as the amount of total solid added is increased. If such curves are found one may be sure that the preparation is impure. On the other hand, if a unit slope followed by a horizontal line is obtained, it is an excellent criterion of purity, especially if the same result holds in other solvents. In conjunction with electrophoretic and ultracentrifugal homogeneity, this provides the most satisfactory evidence in our possession for the purity of proteins.

BIOLOGICAL ACTIVITY

Specific biological activity itself must tell us whether it is the almost pure substance or the impurity in which we are really interested. It must be borne in mind that different proteins, from different species, may have similar biological activity (isobiological), although they differ physicochemically and serologically, and can thus be distinguished. A protein molecule may also be capable of undergoing more or less profound alteration which may decrease, increase, destroy or retain a biological activity originally present. From this standpoint great care is necessary in achieving purification lest "the stream be found purer at its source."

Constant biological activity must be considered, from the practical point of view, as a most important criterion of purity of biologically

active proteins if sufficiently accurate methods of assay are available. The activity may depend upon a small portion of the molecule, the prosthetic group, such as iron in hemoglobin, copper in hemocyanin, thyroxin in thyroglobulin, riboflavin in Warburg's yellow enzyme, etc. The same prosthetic group may be a component part of otherwise different proteins which therefore exhibit similar biological activity. Such substances, isobiological in one respect may be heterobiological in others. For example, hog and sheep metakentrin both possess similar biological activity as hormones but can be distinguished from one another by serological means. Also, they differ physicochemically as proteins, having, among other things, different isoelectric points. However, in many proteins, such as insulin, certain enzymes, etc., no prosthetic groups have been identified with their specific biological behavior.

DISCUSSION AND SUMMARY

The judgment of purity of a protein or other substance is a practical matter. Where biologically active substances are concerned the evidence required for reaching a reasonable judgment must come from the biologist, the organic chemist, and the physical chemist. Purity should then be defined in operational terms, the more evidence the better. We should have accurate biological assays from the biologist, if possible; evidence from the chemist regarding constancy of analytical figures for certain specifically significant groups or atoms; evidence from the physical chemist regarding homogeneity in electrophoresis, the ultracentrifuge, and solubility. These factors have been discussed briefly and the principles on which the physicochemical methods are based have been outlined.

If the biologist and physical chemist can agree as to what fraction of a preparation appears to be "the substance" and what appears to be impurity, then the evidence from the physical chemist provides the best criteria of purity within the limitations of the experimental methods.

HORMONES OF THE POSTERIOR LOBE OF THE PITUITARY GLAND

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INTRODUCTION

In the fifty years that have elapsed since the first demonstration of the blood pressure raising action of injected extracts of the posterior hypophysis[1, 2] a remarkable number of biological properties have been attributed to this gland. Besides exhibiting a pressor effect, an aqueous extract of the posterior lobe may be capable of effecting contraction of the musculature of the uterus, intestine, stomach, esophagus and gall bladder; of influencing the secretion of urine, milk, gastric and pancreatic juice and bile, and of exerting various effects upon the heart, respiration, metabolism and pigmentation. It is possible that several of these effects may be due to the presence in crude extracts of substances, like histamine, which are not peculiar to the posterior lobe but extractable from a variety of other animal tissues. Moreover, a few of the actions mentioned may possibly be due to substances which arise in portions of the hypophysis other than the posterior lobe, present as contaminants in posterior lobe extracts. Certain of these effects have been sufficiently well characterized, however, to justify their classification as pharmacological, if not truly hormonal, properties of the posterior lobe itself. The most clearly established pharmacological properties of posterior lobe extracts are the effect upon mammalian blood pressure (pressor action), the effect upon uterine muscle (oxytocic action), and the effect upon the rate of urine excretion (antidiuretic action). It is with these three activities and particularly the pressor and oxytocic activities, that we are concerned in this discussion.

Thus far, the use of the singular or plural of the word hormone has been avoided in referring to the properties of the posterior lobe since at present it is impossible to decide whether only one or more than one active principle exists in the gland under physiological conditions. Furthermore, in the biochemical approaches to the posterior lobe problem, as in most investigations of the endocrines, the gland tissue is subjected to conditions which are far from physiological. Consequently, a dis-

[1] **Oliver, G., & Schäfer, E. A.** Jour. Physiol. **18**: 277. 1895.
[2] **Howell, W. H.** Jour. Exp. Med. **3**: 245. 1898.

tinction must be made between the chemical conditions that prevail in the living gland and those that exist in the dead tissue extracts commonly employed in laboratory investigations. As practically all of the biochemical data to be discussed have been obtained with gland extracts, it may be advisable to make a brief preliminary examination of the evidence to determine, if possible, whether we may consider the several activities of posterior lobe extracts to be the properties of a single molecule or of separate molecules.

No pure crystalline compounds possessing any of the three activities mentioned have been isolated from posterior lobe tissue. However, by fractionation procedures Kamm,[3] Stehle,[4] and du Vigneaud[5] and their respective associates, have obtained non-crystalline preparations of high pressor potency and very low oxytocic activity. Similarly, amorphous preparations of high oxytocic potency and negligible pressor activity have been obtained. Such fractionations can be carried out without destruction or loss of either activity and no qualitative differences can be detected between the pharmacological responses obtained with each of the separate fractions and the corresponding effects exhibited by unfractionated extracts. Additional evidence which favors the separability of the pressor and oxytocic activities can be found in the work of Dudley,[6] Schlapp[7] and Draper[8] who found that butyl alcohol extraction of acid posterior lobe extracts removed oxytocic activity at a much greater rate than it removed the pressor activity. Electrophoresis experiments[9, 10] have shown that the mobility of the pressor activity is many times greater than the oxytocic, both in solutions of purified fractions and in the untreated press juice from fresh posterior lobes. Finally, it has been shown that the substances responsible for the pressor and oxytocic activities possess different isoelectric points.[11]

From these facts it would appear that by suitable treatment of posterior lobe extracts a substance which accounts for the pressor activity can be separated from one which accounts for the oxytocic activity and that each fraction can exert its pharmacological effect independently of the other. Considered in this light it is justifiable to speak of the substances responsible for these actions as the pressor and oxytocic principles or hormones.

[3] **Kamm, O., Aldrich, T. B., Grote, I. W., Rowe, L. W., & Bugbee, E. P.** Jour. Am. Chem. Soc. **50**: 573. 1928.
[4] **Stehle, R. L., & Fraser, A. M.** Jour. Pharmacol. **55**: 136. 1935.
[5] **Irving, G. W., Jr., Dyer, H. M., & du Vigneaud, V.** Jour. Am. Chem. Soc. **63**: 503. 1941.
[6] **Dudley, H. W.** Jour. Pharmacol. **14**: 295. 1919.
[7] **Schlapp, W.** Quart. Jour. Exp. Physiol. **15**: 327. 1925.
[8] **Draper, W. B.** Am. Jour. Physiol. **80**: 90. 1927.
[9] **du Vigneaud, V., Irving, G. W., Jr., Dyer, H. M., & Sealock, R. R.** Jour. Biol. Chem. **123**: 45. 1938.
[10] **Irving, G. W., Jr., & du Vigneaud, V.** Jour. Biol. Chem. **123**: 485. 1938.
[11] **Cohn, M., Irving, G. W., Jr., & du Vigneaud, V.** Jour. Biol. Chem. **137**: 635. 1941.

Evidence concerning the antidiuretic effect is far from convincing but there is a possibility that this effect may also be due to a separate principle. Kamm[3] found that antidiuretic activity was extremely low in his purified oxytocic fractions but was present in large amounts in potent pressor preparations. This indicated that the oxytocic principle was probably not concerned with antidiuretic activity. Moreover, Gilman and Goodman[12] claim that antidiuretic activity is resistant to the action of certain reducing agents which destroy oxytocic activity. Heller[13] has shown that during heat inactivation over a wide pH range (0.57 to 10.0), pressor activity is lost slightly more rapidly than antidiuretic activity, indicating that these two effects may be due to different agents. Until more data are available, conclusions regarding the status of the substance responsible for antidiuretic activity must be withheld.

On the basis of the evidence outlined above, workers have come to regard the pressor and oxytocic activities as manifestations of two separate hormones. It should be emphasized, however, that none of this evidence precludes the possibility that the separate hormones may be linked together in the form of a single, composite molecule, in the gland itself or even in aqueous gland extracts. The proof of the existence of such a "mother-molecule" rests upon its isolation and the demonstration that it not only accounts for all of the pharmacological properties of the gland, but also lends itself to fragmentation under the relatively mild conditions employed for the fractional separation of the pressor and oxytocic principles. Professor Abel and his collaborators at Johns Hopkins long maintained that the activities of the posterior lobe were properties of a large, labile "mother-molecule" but they were unable to secure conclusive proof.[14] In recent months, however, van Dyke and his associates at the Squibb Institute have gone a long way toward achieving this goal.[15] These investigators have isolated from the posterior lobe of oxen a protein which appears to be homogeneous and which possesses pressor, oxytocic and antidiuretic activity. Ultracentrifugal data obtained by Rosenfeld[16] also indicate that the pressor and oxytocic activities in the press juice from fresh posterior lobes may be associated with a rapidly sedimenting substance, presumably a protein, which is not present in purified preparations of the two principles.

From this brief survey it is clear that it is possible to obtain from extracts of the posterior lobe separate chemical entities which are respon-

[12] **Gilman, A., & Goodman, L.** Jour. Physiol. **90**: 113. 1937.
[13] **Heller, H.** Jour. Physiol. **96**: 337. 1939.
[14] **Abel, J. J.** Jour. Pharmacol. **40**: 139. 1930.
[15] **van Dyke, H. B., Chow, B. F., Greep, R. O. & Rothen, A.** Am. Jour. Physiol. **133**: 473. 1941; Abstracts, 102nd Meeting Am. Chem. Soc. 1941, p. B-10.
[16] **Rosenfeld, M.** Bull. Johns Hopkins Hosp. **66**: 398. 1940.

sible for the oxytocic and pressor activities, respectively; but it is not clear whether or not these separate entities exist as such in the gland and in certain types of extracts made from the gland. As we have pointed out, recent work makes it necessary seriously to consider anew the possibility of the existence of a "mother-molecule" which possesses both activities, and which is cleaved in the course of extraction or fractionation. We shall consider this point in greater detail later in this report.

In discussing the chemistry of the posterior lobe we are concerned with a review of the properties and behavior of the several active, amorphous preparations that have been isolated from the gland by various procedures. First we shall discuss very briefly the assay techniques used in these investigations. Next we shall discuss the methods of preparation and the chemical and physical properties of the separate pressor and oxytocic preparations. Finally, we shall make a similar survey for the protein isolated by van Dyke and his associates. With this information before us we may be in a position to make further comment upon the unitary and multiple hormone concepts.

ASSAY METHODS

Pressor Assay

The development of the pressor assay method is largely due to the work of Hamilton and Rowe.[17-19] The test animal is the anesthetized dog or cat. Changes in blood pressure in a cannulated carotid artery are registered by means of a mercury manometer and recorded on a smoked drum of the familiar kymograph. The solution to be assayed is injected into a leg vein and, after an interval of 15 minutes, is followed by an injection of the reference standard. Alternate injections of the unknown, in increasing amounts, and the standard are made at 15 minute intervals (to avoid tachyphylaxis) until the blood pressure rise due to the unknown is approximately equal to that produced by the standard in at least two successive pairs of responses. Since the pressor content of the standard dose is known, the ratio between the heights of the standard and unknown responses gives directly the pressor content of the volume of unknown injected. As used routinely, the accuracy of the method is hardly better than $\pm$ 20 per cent.

The pressor standard used is a powdered preparation of desiccated fresh beef posterior lobes (Standard Powder) which contains 2 International Pressor Units per mg. when prepared as prescribed by the U. S.

[17] **Hamilton, H. C.** Jour. Am. Pharm. Assoc. **1**: 1117. 1912.
[18] **Hamilton, H. C., & Rowe, L. W.** Jour. Lab. Clin. Med. **2**: 120. 1916.
[19] **Rowe, L. W.** Endocrinology **13**: 205. 1929.

Pharmacopoeia. This standard was originally established by the Conference on Biological Standardization of the League of Nations to serve as the oxytocic standard[20] but has since been adopted as the standard for pressor and antidiuretic assay.[21] It is agreed that 1 mg. of the Standard Powder contains 2 units of each of the three activities.

The pressor assay dose varies between 0.1 and 0.5 unit (0.05 to 0.25 mg. of Standard Powder). Since this small amount of material usually produces a blood pressure rise of approximately 10 mm. of mercury, the pharmacological effectiveness of the pressor principle is apparent. This effectiveness is better appreciated when it is realized that only 0.5 to 2.5 micrograms of a purified pressor preparation (200 units per mg.) is required to produce the same response.

Oxytocic Assay

Two methods are available for oxytocic assay. One of these involves the measurement of contraction in an isolated strip of uterus of the guinea pig. The second method involves the measurement of the fall in blood pressure in the fowl.

The uterine strip procedure was suggested and developed by Dale and Laidlaw[22] and was later modified by Burn and Dale.[23] The official method of the U. S. Pharmacopoeia X is essentially a refinement of their original technique.

One horn of a virgin guinea pig uterus is suspended in a constant 37° C. bath of a balanced physiological salt solution (usually Ringer-Locke solution). One end of the strip is fixed while the free end is fastened to a light lever which registers the extent of movement on a kymograph drum. The solution to be assayed and the standard are added to the bath alternately in varying doses until quantities of the two solutions are found which give equal, sub-maximal contractions in at least two successive pairs of responses. After each contraction the bath solution is discarded and renewed. The oxytocic activity of the unknown is calculated from the ratio between the unknown and standard responses. The accuracy is $\pm$ 20 per cent.

The depressor action of pituitary extracts upon the blood pressure of decapitated ducks was first observed by Paton and Watson.[24] Subsequent investigation has shown that the effect can also be produced in anesthetized fowls and that it is due to the oxytocic principle. This

[20] **League of Nations,** Report by the Second International Conference on Biological Standardization of Certain Remedies. Geneva. August, 1925. p. 14.
[21] **League of Nations.** Quart Bull. of the Health Organization **5**: 572, 582. 1936.
[22] **Dale, H. H., & Laidlaw, P. P.** Jour. Pharmacol. **4**: 75. 1912.
[23] **Burn, J. H., & Dale, H. H.** Med. Res. Counc. Spec. Rep. Series **No. 69.** 1922.
[24] **Paton, D. N., & Watson, A.** Jour. Physiol. **44**: 413. 1912.

effect has recently been utilized by Coon,[25] working in the laboratory of Professor Geiling at the University of Chicago, in developing the fowl blood pressure method for oxytocic assay.

Blood pressure changes in a cannulated leg artery (ischiatic) of an anesthetized hen or rooster are recorded in a manner similar to that used in the pressor assay. The solution to be assayed is injected into an exposed leg vein (crural) and it is followed, after a 3 to 5 minute interval, by an injection of the reference standard. Alternate injections of standard and unknown are made in this manner until closely similar depressor responses are obtained. The oxytocic activity of the injected dose of unknown is then calculated from the ratio between these two responses. Reproducibility and accuracy are as good with the fowl blood pressure method as with the uterine strip technique and the former offers the advantage of being a less temperamental test subject. The two methods agree well in the assay of unknowns provided the pressor: oxytocic ratio does not exceed 2.5. However, Coon argues that there is no reason to suspect the fowl blood pressure values as being the incorrect ones in such cases.

The oxytocic standard is the previously described Standard Powder containing 2 oxytocic units per mg. In the uterine strip method a dose of 0.2 unit produces a contraction of several mm. In the fowl a similar dose (0.4 to 0.8 micrograms of a purified oxytocic preparation) produces a blood pressure drop of 20 to 40 mm. The enormous pharmacological effectiveness of the oxytocic principle is obvious.

Antidiuretic Assay

The usual method for antidiuretic assay is similar to that of Gibbs[26] as modified by Burn.[27] Five cc. of water per 100 gm. body weight are administered by stomach tube or intraperitoneal injection to each of several rats and the solution to be assayed is injected into each animal. The rats are caged together and the time required for the rate of urine excretion to reach a maximum, or the time required for the excretion to attain 50 per cent, is determined. Another group of rats is treated similarly except that a known amount of standard is injected instead of the unknown. The ratio between the standard and unknown time intervals serves as the basis for calculating the antidiuretic activity of the unknown. The error is usually less than 20 per cent if precautions are taken to rule out the individual variations between groups of test animals.

The assay dose is approximately 0.006 unit per 100 gm. body weight.

[25] **Coon, J. M.** Arch. inter. Pharmacodynamie **62**: 79. 1939.
[26] **Gibbs, O. S.** Jour. Pharmacol. **40**: 129. 1930.
[27] **Burn, J. H.** Quart. Jour. Pharm. **IV**: 517. 1931.

This dose reduces the rate of urine excretion to about 50 per cent of that found in an untreated animal. Since the method is capable of differentiating doses differing by as little as 0.002 unit (approximately 1 microgram of Standard Powder), the weight of purified fractions required to produce a similar response is vanishingly small.

PURIFICATION OF THE PRESSOR AND OXYTOCIC PRINCIPLES

Procedures for the purification of the active principles have depended, in general, upon the solubility of the principles in water or aqueous solvents and their relative insolubility in organic solvents and in concentrated salt solutions. However, the principles are soluble to some extent in the lower alcohols, particularly if small amounts of water are present, and this solubility increases markedly as the principles are purified. In some cases solubility in organic solvents is also increased by the presence of acid. In partially purified preparations, the oxytocic principle is more soluble than the pressor in certain organic solvents. Use has been made of this property in procedures for separating these two principles. The principles are appreciably soluble in a few of the lower molecular weight fatty acids, acetic acid being the best of this series. Various organic acids and metallic salts as well as numerous adsorbents have been used with varying degrees of success for the purification and separation of the principles. Unfortunately, separation of the active material and the reagent, or elution of the active material from the adsorbent, usually results in sufficient loss or destruction of activity to discourage extensive use of such procedures. Recently, however, Potts and Gallagher[28] have used chromatographic adsorption on artificial zeolites as a means for separation and purification of the pressor and oxytocic principles. No details have been published but it appears that separate pressor and oxytocic preparations having potencies much higher than any previously reported have been obtained by this procedure.

For the initial gland extract dilute acetic acid (0.05 to 0.5 per cent) has been favored since it is a good solvent and since the pH of the resulting solution lies close to the pH of maximum stability (pH 3.0).

Three methods for obtaining separate pressor and oxytocic preparations, each adaptable to large scale work, will be described to illustrate the steps involved, the yields obtained and the potencies of the products. The steps involved in these three procedures are shown schematically in TABLES 1–3. For comparative purposes the yields given have been

[28] **Potts, A. M., & Gallagher, T. F.** Proc. Soc. Biol. Chem., Jour. Biol. Chem. **140**: p. ciii. 1941. Jour. Biol. Chem. **143**: 561. 1942.

recalculated to show the amounts of purified pressor and oxytocic fractions which can be obtained from 1 kg. of dry posterior lobes or its equivalent in fresh gland tissue. Potencies of products are expressed in terms

TABLE 1

PURIFICATION PROCEDURE OF KAMM AND COWORKERS[3]

Fraction	Weight	Procedure	Potency, Units per mg.		Total activity	
			Pressor	Oxytocic	Pressor	Oxytocic
	gm.				*Per cent*	*Per cent*
A	1000	Acetone desiccated posterior lobes Extracted with hot 0.25 per cent HAc; concentrated; saturated with $(NH_4)_2SO_4$. ↓	1	1	100	100
B		Precipitate, dried Extracted with glacial HAc; ether and petroleum ether added to extract. ↓				
C	68	Precipitate Dissolved in 98 per cent HAc; ether added; repeated once.	11	12	75	82
D	63	Precipitate Dissolved in 98 per cent HAc; fractionally precipitated with acetone and ether to give several precipitates.	10	7	63	44
E	5.9	Most potent precipitate Dissolved in 98 per cent HAc; fractionally precipitated with acetone and ether as above.	62	14	37	8.3
F (Pressor)	2.0	Most potent precipitate ↓ HAc-ether mother-liquor treated with trace of water and excess of petroleum ether. ↓	80	15	16	3.0
G (Oxytocic)	2.4	Gummy precipitate	6	160	1.5	38

of International Units per mg. of solid. It should be noted that as these figures represent averages in most cases, the yields and potencies of the products obtained in individual experiments will vary slightly from those given.

The figures in TABLES 1–3 indicate that the most potent pressor preparations contain approximately 200 units per mg. Such preparations also contain small amounts of oxytocic activity. As mentioned previously, however, the accuracy of oxytocic assays in the presence of such large amounts of pressor activity is open to question. Moreover, the

TABLE 2
PURIFICATION PROCEDURE OF STEHLE AND FRASER[4]

Fraction	Weight	Procedure	Potency, Units per mg.		Total, Activity	
			Pressor	Oxytocic	Pressor	Oxytocic
	gm.				*Per cent*	*Per cent*
A	1000	Acetone desiccated posterior lobes Extracted with hot 0.5 per cent HAc; concentrated; treated with absolute ethanol.	1	1	100	100
B	218	Alcohol precipitate Dissolved in 0.5 per cent H_2SO_4; $Ba(OH)_2$, $Fe_2(SO_4)_3$ and dialysed iron added; filtrate concentrated to dryness; dissolved in 96 per cent methanol; fractionated with EtAc, repeated twice.	4	4	87	87
C	7.3	Precipitate Fractionated from ethanol or methanol with EtAc.	100	30	73	22
D (Pressor)	2.0	Precipitate Methanol-EtAc mother-liquor fractionated with methanol, ethanol and EtAc.	200	10	40	2
E (Oxytocic)	0.55	Precipitate	4	250	0.2	14

TABLE 3

PURIFICATION PROCEDURE OF IRVING, DYER, AND DU VIGNEAUD[5]

Fraction	Weight	Procedure	Potency, Units per mg.		Total Activity	
			Pressor	Oxytocic	Pressor	Oxytocic
	gm.				*Per cent*	*Per cent*
A	6000	Frozen posterior lobes Ground with sand and water; juice separated under pressure; juice acidified, boiled 10 min., centrifuged; supernatant concentrated, saturated with NaCl.	0.2	0.2	100	100
B	200	Precipitate, dried Extracted with 98 per cent HAc; ether and petroleum ether added to extract.	4	4	80	80
C	78	Precipitate Dissolved in 98 per cent HAc; ether added; repeated once.	9	9	72	75
D	58	Precipitate Dissolved in 98 per cent HAc; fractionally precipitated with acetone and ether to give several precipitates.	9	5	52	27
E	5.2	Most potent precipitate Dissolved in 98 per cent HAc; fractionally precipitated with acetone and ether as above.	55	30	29	16
F	1.5	Most potent precipitate Dissolved in glacial HAc, precipitated with ether, repeated 3 times.	85	30	13	4.5
G	1.1	Precipitate Dissolved in water; extracted with butanol.	110		12	
H	0.6	Aqueous solution Electrophoresis and isolation of cathode material.	125	20	8.1	1.3
I (Pressor)	0.2	Precipitate HAc-ether mother-liquor treated with water and petroleum ether.	200	26	4.0	0.5
J		Gummy precipitate	22	146	5	24

possibility cannot be ignored that the pressor principle itself may possess slight inherent oxytocic properties. A similar possibility must also be admitted regarding the presence of pressor activity in oxytocic preparations. The most potent oxytocic preparation obtained has an activity of 250 units per mg. By procedures not fully described, Kamm and his coworkers[3] state that they were able to prepare an oxytocic fraction having an activity of 350 units per mg. In a later paper[29] it was indicated that potencies as high as 500 oxytocic units per mg. had been attained. The pressor and oxytocic preparations described recently by Potts and Gallagher[25] contain 450 and 700 units per mg., respectively.

The purified pressor and oxytocic preparations are white or slightly cream colored, amorphous powders. Hygroscopicity varies but is usually insufficient to cause trouble in weighing or handling under ordinary conditions. The activity of these dry preparations is quite stable for periods of at least several months.

CHEMICAL AND PHYSICAL PROPERTIES OF THE SEPARATE PRESSOR AND OXYTOCIC PREPARATIONS

Analysis

The first significant analytical study of posterior lobe hormone preparations was undertaken by du Vigneaud and his associates in 1933[29] when it was shown that these preparations contain relatively large amounts of cystine sulfur and tyrosine. These data together with analytical figures obtained subsequently in other laboratories are given in TABLE 4. Data are included for several purified pressor and oxytocic preparations and for a few preparations of low potency. The differences between the analytical values for a given constituent may appear to be great but, inasmuch as the materials analysed were prepared by different procedures and the determinations made by different methods, such variations are not surprising. The figures indicate that highly purified pressor preparations contain large amounts of cystine, tyrosine and arginine, while highly purified oxytocic preparations contain closely similar amounts of cystine and tyrosine but considerably less arginine. Unfortunately, the preparations analysed are not pure substances. Consequently, the analysis of a few preparations provides no sound basis for predicting the composition of the principles themselves.

Very limited but more indicative information concerning the probable composition of the principles has been gained by the systematic analysis

[29] **du Vigneaud, V., Sealock, R. R., Sifferd, R. H., Kamm, O., & Grote, I. W.** Proc. Soc. Biol. Chem., Jour. Biol. Chem. **100**: p. xciv. 1933.

TABLE 4
ANALYSIS OF PRESSOR AND OXYTOCIC PREPARATIONS

Potency of Preparation		N	S	NH_2-N	Cystine	Tyrosine	Arginine	Investigators	Remarks
Pressor	Oxytocic								
Units per mg.	*Units per mg.*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*		
200	?	—	3.1	—		10.5		du Vigneaud and co-workers[29]	Values corrected for ash and moisture.
?	500	—	3.1	—	9.0	14.3			
200	6	15.6	3.4	—		11.5	3.8	Sealock[30]	Values corrected for ash and moisture.
2	500	15.2	3.3	—	9.6	15.8			
200	8–10	13.9	3.0	2.0	7.7	9.5	8.9	Stehle and Fraser[4]	Moisture and ash corrections not indicated. Average values.
4	250	13.8	3.6	2.5	8.9	10.7	6.1		
4	4	13.7	1.6	1.2		2.6	8.6		
200	25	14.3	—	—	11.2	9.9		Irving, Dyer, and du Vigneaud[5]	Not corrected for ash or moisture.
9	9	14.6	—	—	10.8	2.2			
?	240	14.0	3.2	1.4	—	—	—	Freudenberg, Weiss, and Biller[31]	Moisture and ash corrections not indicated.
450	<40	—	—	—	19.0	11.9	12.3	Potts and Gallagher[28]	Moisture and ash corrections not indicated.
<20	700	—	5.6	—	18.3	14.2	<0.8		

[30] **Sealock, R. R.** A thesis submitted to the George Washington University. 1935.
[31] **Freudenberg, K., Weiss, E. & Biller, H.** Z. physiol. Chem. **233**: 172. 1935.

of preparations of different potencies. Data obtained in this manner for both pressor and oxytocic preparations show that the sulfur content of these preparations increases progressively as the pressor or oxytocic potency increases, indicating that both principles undoubtedly contain sulfur.[29] Likewise analytical figures for tyrosine and cystine strongly indicate the presence of these two amino acids in the pressor principle[5] as shown in FIGURE 1. Similar analyses for arginine in both principles

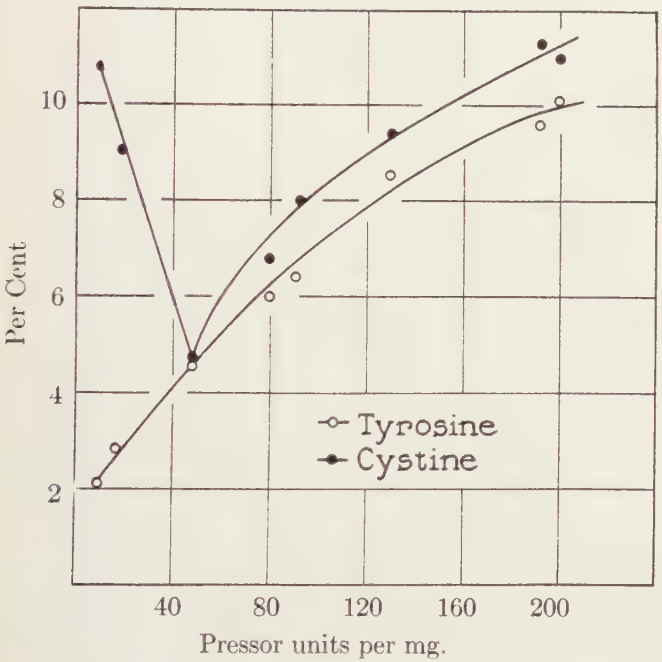

FIGURE 1.

and for cystine and tyrosine in the oxytocic principle have not been carried out.

Stehle and Trister[32] have recently examined purified pressor and oxytocic preparations for a large number of amino acids. These data are tabulated in TABLE 5. In this work, rather large amounts of each of the purified preparations were hydrolyzed in 20 per cent HCl, and attempts were made to isolate certain amino acids or their derivatives from the hydrolysates. Where the results were positive, the yields were sufficiently large to permit purification and characterization of the pro-

[32] **Stehle, R. L., & Trister, S. M.** Jour. Pharmacol. **65**: 343. 1939.

TABLE 5

IDENTIFICATION OF AMINO ACIDS IN HYDROLYSATES OF PRESSOR AND OXYTOCIC PREPARATIONS[32]

Amino acid	Characterized by	Pressor preparation	Oxytocic preparation
Tyrosine	Picrolonate	+	+*
Cystine	Amino acid, hydantoin	+	+*
Arginine	Flavianate	+	+
Proline	Reineckate	+	+
Isoleucine	Hydantoin	+	−
Leucine	Hydantoin	−	+
Histidine	Colorimetric test	−	−*
Glutamic acid	Hydrochloride	−	−
Phenylalanine	Picrolonate	−	−
Hydroxyproline	Reineckate	−	−
Glycine	Orthophthalic aldehyde	−	−
Tryptophane	Colorimetric test	trace*	trace
Cysteine	Colorimetric test	−	−

* Freudenberg and coworkers[31] found tyrosine, cystine and histidine in the hydrolysate of an oxytocic preparation (240 units per mg.).

duct. Qualitative tests were also applied in some cases. These results show that tyrosine, cystine, arginine, and proline can be isolated from hydrolysates of both pressor and oxytocic preparations. Isoleucine seems to be present only in the pressor fraction while leucine appears to be present only in the oxytocic fraction. Stehle and Trister failed to find significant amounts of histidine, glutamic acid, phenylalanine, hydroxyproline, glycine, or tryptophane in either preparation. No tests have been made to determine the presence or absence of alanine, aspartic acid, hydroxyglutamic acid, lysine, methionine, serine, threonine, or valine. With respect to the positive results in TABLE 5, it should be re-emphasized that since the purity of the preparations examined has not been established, inferences regarding composition of the principles themselves must be made with caution.

Freudenberg and Biller[33] have reported the isolation of choline as the reineckate from a sulfuric acid hydrolysate of an oxytocic preparation containing 250 oxytocic units per mg. and estimate from the yield that more than 1 per cent of choline could be present in the oxytocic principle. Prompted by this observation, and the hypothesis that the oxytocic principle might be a choline ester of an amino acid or peptide, Freudenberg and Keller[34] and later Gulland, Partridge and Randall[35] synthesized many choline esters of this type but, so far, none of the synthetic products exhibits significant oxytocic activity. Apparently the possibility was not

[33] **Freudenberg, K., & Biller, H.** Naturwissensch. **24**: 523. 1936.
[34] **Freudenberg, K., & Keller, R.** Ber. **71B**: 329. 1938.
[35] **Gulland, J. M., Partridge, M. W. & Randall, S. S.** Jour. Chem. Soc. p. 419. 1940.

considered that the choline might have been present as an impurity in the original oxytocic preparation.

In purified pressor preparations choline is absent.[36] We have found that hydrolysates of highly purified pressor fractions exhibit a powerful depressor action upon mammalian blood pressure, a fact which is suggestive of choline. This action is not appreciably increased by acetylation nor is it blocked by atropinization under conditions in which controls with added choline respond in the expected manner. Furthermore, injections of large amounts of an unhydrolyzed pressor fraction into an animal which has been made refractory to the pressor principle by rapidly repeated injections, produce a depressor response that is qualitatively and quantitatively identical with that obtained by using an equivalent amount of hydrolysate of the same pressor fraction. In other words, the depressor action of the unhydrolyzed material becomes evident when the masking effect of the predominant pressor action is eliminated either by hydrolysis or by test upon a pressor-refractory animal. In our opinion these observations show conclusively that the substance responsible for the depressor action of hydrolyzed pressor preparations is not choline and further, that this substance is not an integral part of the pressor molecule. Tests upon fractions obtained at various stages in the purification of the pressor principle show that this depressor impurity is present in relatively large amounts in crude gland extracts and that it is partially eliminated in the purification process. From its solubility and pharmacological behavior it is likely that the substance may be histamine, which has been isolated from posterior lobe extracts.[37] Only 0.01 mg. of histamine per 100 mg. of potent pressor preparation could account for the depressor effects observed.

A similar study of potent oxytocic preparations would be of definite value in clarifying the present status of the oxytocic principle with respect to choline.

Molecular Weight

With the possible exception of choline, significant amounts of substances other than amino acids have not been found in hydrolysates of either principle. Furthermore, elementary analysis indicates the presence of approximately 15 per cent nitrogen and 3 per cent sulfur. These facts, together with the information concerning solubility behavior gained from purification studies, have naturally led to the supposition that the principles themselves are either proteins or polypeptides. Con-

[36] **du Vigneaud, V., & Irving, G. W., Jr.** Unpublished data.
[37] **Abel, J. J., & Kubota, S.** Jour. Pharmacol. **13**: 243. 1919.

siderable evidence is available to substantiate this view. Positive biuret and ninhydrin color reactions are obtained with purified preparations of both principles. Acid hydrolysis rapidly destroys pressor, oxytocic and antidiuretic activity with the simultaneous liberation of amino acids. Hydrolysis of potent pressor and oxytocic preparations results in a four- to fivefold increase in amino nitrogen. Certain proteolytic enzymes rapidly destroy all three activities.

It may be concluded, therefore, that the molecules responsible for the activities of pressor and oxytocic preparations contain amino acids joined in peptide linkage, and that the presence of the intact peptide structure is essential for the pharmacological activity of these molecules. Since it is obvious that either a peptide or a protein could satisfy these requirements additional evidence must be examined to determine the approximate size of the active molecules.

As early as 1899 Schäfer and Vincent[38] discovered that pressor activity is not retained by dialyzing membranes. From this observation, which has been confirmed and extended to include the oxytocic and antidiuretic activities, it is evident that the size of the molecules responsible for these activities is of a much lower order of magnitude than even the simplest proteins. Approximations of molecular size have been obtained by comparing the rate of dialysis of the principles with the rates for substances of known molecular weight. Smith and McClosky[39] found that all three activities passed rapidly through a collodion membrane which was completely impermeable to trypan blue (mol. wt. 932). The diffusion rates for the two principles were approximately equal and agreed closely with the rate for methylene blue (mol. wt. 374). Kamm[40] found that the pressor and oxytocic principles diffused through collodion at about half the rate of adrenalin (mol. wt. 183) and estimated the molecular weights of each of the two principles to be in the neighborhood of 600. On the basis of ultracentrifuge studies Rosenfeld[10] agrees that Kamm's estimate is probably of the correct order of magnitude, since the pressor and oxytocic activities contained in commercial preparations of the two principles (pitressin and pitocin), showed but little tendency to sediment after nearly 6 hours of centrifuging at 61,000 r.p.m. Molecular weight estimates have also been made from analytical data. Stehle and Fraser[1] find that the simplest molecular weights which can be calculated from the sulfur contents of the purified principles, assuming that the sulfur is present as cystine sulfur, are 1778 for the oxytocic and 2160 for the pressor principle. Assuming 1 amino group per molecule a

[38] **Schäfer, E. A., & Vincent, S.** Jour. Physiol. **25**: 87. 1899.
[39] **Smith, M. I., & McClosky, W. T.** Jour. Pharmacol. **24**: 371. 1924.
[40] **Kamm, O.** Science **67**: 199. 1928.

molecular weight of 700 can be calculated from Stehle and Fraser's figures which indicate the presence of approximately 2.0 per cent amino nitrogen in the unhydrolyzed preparations. Potts and Gallagher[28] find that the cystine and tyrosine values for their 700 unit oxytocic fraction are in good agreement with a minimum molecular weight of 1300 for the oxytocic principle, assuming the presence of one molecule of each amino acid. On the same basis, a closely similar minimum molecular weight can be calculated for their 450 unit pressor fraction.

The evidence presented so far indicates (1) that the two active principles are polypeptides; (2) that they have molecular weights between 600 and 2000; and (3) that they probably contain cystine, tyrosine, and arginine and possibly proline and leucine or isoleucine. Aside from the very significant fact that the highly purified oxytocic preparation of Potts and Gallagher[28] seems to contain considerably less arginine than their purest pressor fraction none of the evidence presented so far reveals any striking chemical or physical differences between the two principles nor does it indicate what parts of the molecules are necessary for their pharmacological activity. A number of approaches have been used in an attempt to throw additional light upon these questions.

Action of Enzymes

The action of proteolytic enzymes has been investigated almost entirely from the standpoint of their effects upon the activity of purified preparations. Trypsin preparations rapidly destroy pressor, oxytocic and antidiuretic activity whereas pepsin has no effect upon any of them.[41–47] Results with papain are in disagreement but it is probable that this enzyme destroys oxytocic activity.[44,45,48] Extracts of kidney, liver, muscle, blood, yeast, and intestinal mucosa destroy pressor and oxytocic activity.[45,49] Gulland finds that aminopolypeptidase, dipeptidase, and proteinase preparations from yeast destroy oxytocic activity, but believes that the action is due to a separate enzyme present in each of these preparations and also present in trypsin preparations. Larson[49] believes that aminopolypeptidase alone is responsible for the destruction of pressor activity, dipeptidase being without effect.

Besides establishing the inactivating effect of enzymatic hydrolysis of peptide bonds, these observations provide little information concerning

41 **Dale, H. H.** Biochem. Jour. **4**: 427. 1909.
42 **Dudley, H. W.** Jour. Pharmacol. **14**: 295. 1919.
43 **Thorpe, W. V.** Biochem. Jour. **20**: 374. 1926.
44 **Freudenberg, K., Weiss, E., & Eyer, H.** Naturwissensch. **20**: 658. 1932.
45 **Gulland, J. M., & Macrae, T. F.** Nature **131**: 470. 1933.
46 **Gulland, J. M., & Macrae, T. F.** Biochem. Jour. **27**: 1237. 1933.
47 **Gulland, J. M., & Macrae, T. F.** Biochem. Jour. **27**: 1383. 1933.
48 **Dale, H. H., & Dudley, H. W.** Jour. Pharmacol. **18**: 27. 1921.
49 **Larson, E.** Jour. Pharmacol. **62**: 346. 1938.

the nature of the active molecules, but the difference between the action of trypsin and pepsin suggests that more might be learned by this approach. A careful study of the pituitary principles with a number of specific proteolytic enzymes might reveal certain structural possibilities or differences.

Other enzymatic studies indicate that prolinase and arginase exert no effect upon the activity of the oxytocic principle,[57] but tyrosinase causes complete inactivation.[58] This observation points to the possible essentiality of the tyrosine phenolic grouping to the activity of the oxytocic principle.

Action of Various Chemical Agents

Investigation of the effects of various chemical and physical treatments upon the activity of the pressor and oxytocic principles has provided material for speculation but very little concrete information as to the structure of the hormones themselves, as shown in TABLE 6.

It is well known that the principles are quite stable in dilute acid (pH 3.0).[59] At this pH the principles may be boiled for several minutes without affecting their activity. It is equally well recognized that the principles are quite unstable in alkaline solution. Exposure to 1 N alkali for a few hours at room temperature is sufficient to destroy completely both pressor and oxytocic activity[42], and more vigorous treatment with alkali is accompanied by the liberation of hydrogen sulfide.[1]

The action of many chemical agents upon the activity of purified oxytocic preparations has been extensively studied in the laboratories of Kamm,[2,3] Guha[54,55] and Gulland[59,60], but the experimental conditions used have varied so widely that it is hazardous to base any conclusions regarding chemical structure upon an analysis of the assembled data. Under these circumstances it is preferable to present the authors' interpretations as to the significance of their results.

Kamm and coworkers were of the opinion that such reagents as Na_2SO_3, $NaHSO_3$, $Na_2S_2O_3$, $Na_2S_2O_4$ and SO_2 not only destroy pressor and oxytocic activity but that they convert the pressor principle into a

[50] **Gaddum, J. H.** Biochem. Jour. **24**: 939. 1930.
[51] **Sullivan, M. X., & Smith, M. I.** Public Health Reports. U. S. P. H. S. **43**: 1334. 1928.
[52] **Kamm, O., Grote, I. W., & Rowe, L. W.** Proc. Soc. Biol. Chem., Jour. Biol. Chem. **92**: p. lxix. 1931.
[53] **Kamm, O., & Grote, I. W.** Canadian Patent 367,624, July 27, 1937, Chem. Abs. **31**: 6823. 1937.
[54] **Guha, B. C., & Chakravorty, P. N.** Indian Jour. Med. Res. **21**: 429. 1933.
[55] **Das, N., & Guha, B. C.** Indian Jour. Med. Res. **21**: 765. 1934.
[56] **Das, N., & Guha, B. C.** Indian Jour. Med. Res. **22**: 157. 1934.
[57] **Das, N., & Guha, B. C.** Indian Jour. Med. Res. **22**: 517. 1935.
[58] **Gulland, J. M.** Biochem. Jour. **27**: 1218. 1933.
[59] **Gulland, J. M., & Randall, S. S.** Biochem. Jour. **29**: 378. 1935.
[60] **Gulland, J. M., & Randall, S. S.** Biochem. Jour. **29**: 391. 1935.

"derived" hormone having a very low sulfur content and possessing antidiuretic activity. The chemical changes involved were not discussed nor have the experimental findings been confirmed. Gilman and Goodman[12] mention the fact that treatment with sodium sulfite does not cause loss of antidiuretic activity.

Guha and his collaborators tested the effects of H_2O_2, HNO_2, HNO_3, Br_2, SO_2, and benzoyl and acetyl chlorides upon the activity of the oxytocic principle but refrained from discussing the structural implications of their results. Inactivation was obtained in all cases but the absence of adequate controls makes their estimates of the degree of inactivation questionable.

TABLE 6

ACTION OF VARIOUS AGENTS UPON PURIFIED PRESSOR AND OXYTOCIC PREPARATIONS

Agent	Pressor	Oxytocic
Acid hydrolysis	Inactivated	Inactivated
Alkali, cold	"	"
Na_2SO_3	"	"
$NaHSO_3$	"	?
$Na_2S_2O_3$	"	?
$Na_2S_2O_4$	"	?
HNO_2	?	Inactivated
NaCN	?	"
H_2O_2	?	"
I_2, Cl_2, Br_2	?	"
Ultraviolet light	?	"
SO_2	Inactivated	Partial to complete inactivation
HCN	?	Partially inactivated
Benzoyl chloride	?	" "
Acetyl chloride	?	" "
Electrolytic reduction	?	Partially inactivated (irreversible)
Catalytic reduction	?	Partially inactivated (irreversible?)
H_2S	?	Partially inactivated (reversible)
Semicarbazide	?	Unaffected
Hydroxylamine	?	"
Cysteine reduction	Unaffected	"
Cysteine reduction followed by benzylation	Inactivated	Inactivated

Gulland and coworkers observed that HNO_2 completely inactivates the oxytocic hormone and believed that three separate reactions are involved in the inactivation process. In the first step nitrous acid is presumed to convert the principle rapidly into a derivative possessing 35 per cent of the original potency. This derivative is then converted into a second intermediate having a potency of 20 per cent and finally

into a substance having no oxytocic activity. No chemical changes were suggested to explain this behavior.

Gulland and Randall found that inactivation by reduction could be reversed in some cases by oxidation but that the degree of reactivation depended largely upon the reducing conditions used and the extent of the reduction. Thus, an oxytocic preparation which had been 50 per cent inactivated through the action of H_2S for 24 hours could be completely reactivated by treatment with oxidized methylene blue at pH 4.2. On the other hand, when the same degree of reductive inactivation was accomplished catalytically or electrolytically, treatment with oxidized methylene blue caused only partial reactivation. By using a series of oxidation-reduction dyes the oxytocic principle was found to contain a redox system having a potential of $E_0' = +0.025$ volts at pH 6.0. When the system is completely oxidized the hormone is considered to be fully active; when fully reduced the activity is 50 per cent. Attempts were made to elucidate the structure of this redox system by studying the effects of the following reagents upon oxytocic activity: Na_2SO_3 at pH 8.4, SO_2 at pH 5.5, NaCN at pH 7.8, HCN at pH 3.5, H_2O_2 at pH 3.5 and 8.2, I_2 at pH 7.5, Cl_2 in acid solution, semicarbazide, and hydroxylamine. Although these workers were unable to correlate their data with any definite structure they suggested that none of the results was opposed to the view that a disulfide linkage could be present in the hormone molecule if the corresponding reactions of cystine were used as an analogy. Thus, cystine is reduced by Na_2SO_3 and by NaCN and these compounds inactivate the oxytocic principle. Similarly, cystine is oxidized to cysteic acid by iodine and chlorine and the halogens also inactivate the hormone. Gulland and Randall are forced to admit, however, that SO_2 and HCN, which exert no effect upon cystine, both cause appreciable inactivation. Furthermore, H_2O_2 which rapidly oxidizes cystine had a most peculiar action upon the oxytocic principle. In either acid or alkaline solution, H_2O_2 first caused 50 per cent inactivation, then a reactivation to 90 per cent, and finally complete destruction. In view of these facts Gulland and Randall felt that they were not justified in making definite the suggestion that a disulfide linkage is present in the oxytocic molecule.

Gilman and Goodman[12] claim that antidiuretic activity is resistant to the reducing agents found by Gulland and coworkers to inactivate the oxytocic hormone.

The chemical information available in 1935 suggested to Sealock and du Vigneaud[61] certain similarities between the posterior lobe principles

[61] **Sealock, R. R., & du Vigneaud, V.** Jour. Pharmacol. **54**: 433. 1935.

and insulin. With the latter hormone it had been demonstrated[62] that reduction of disulfide linkages in the molecule to the sulfhydryl form by means of cysteine or reduced glutathione resulted in irreversible destruction of hormonal activity. This technique seemed to provide a method for demonstrating whether or not the sulfur of posterior lobe preparations was actually present as a disulfide in the active principles and if so, whether or not reduction of this linkage caused inactivation. Solutions of highly purified pressor and oxytocic preparations, buffered at pH 8.0, were treated with an excess of cysteine at room temperature in an atmosphere of nitrogen for periods of 2 to 48 hours. With both preparations the reduced products and the products obtained by reoxidation were found to be as active pharmacologically as the original preparations. In view of these results proof was needed that reduction by cysteine had been accomplished. It was reasoned that if the principles originally contained disulfide linkages, then the sulfhydryl groups formed on reduction should be susceptible to benzylation or methylation. When the original preparations were treated with benzyl chloride or methyl iodide no change in activity resulted. But when the reduced solutions were treated similarly, until the test for sulfhydryl groups was negative, all pressor and oxytocic activity was destroyed. In the latter experiments assays made at intervals during the benzylation showed that the activity decreased gradually over a 10 hour period, indicating that the inactivation was undoubtedly the result of the benzylation reaction. Suitable controls were run to show that the pH of the medium caused no injury to the active principles and that the reagents used did not affect the assay animals.

The authors felt that the combination of cysteine reduction and benzylation, together with the effects of these treatments on pharmacological activity, offered almost conclusive proof that the active principles contain a disulfide linkage and that a sulfhydryl group or a potential sulfhydryl group is essential to the activity of these hormones.

The validity of these conclusions was questioned by Gulland and Randall[63] who maintained that cysteine had not been adequately established as a specific reducing agent for the disulfide linkage and consequently, results obtained through its use did not necessarily prove the presence of such a linkage. It was claimed further that the behavior of the hormones to reduction and benzylation could be explained on the basis of a number of redox systems, the reduced forms of which might

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[62] **du Vigneaud, V., Fitch, A , Pekarek, E., & Lockwood, W. W.** Jour. Biol. Chem. **94**: 233. 1931.

[63] **Gulland, J. M. & Randall, S. S.** Jour. Soc. Chem. Ind. **55**: 442. 1936.

be susceptible to benzylation with attendant loss of activity. Such reduced groups as -OH and -NH- were suggested as possibilities.

In reply to the criticism of Gulland and Randall, du Vigneaud[61] emphasized the fact that the conclusions drawn from the reduction experiments were not based upon the specificity of the reduction alone but upon the combination of reduction with cysteine and then inactivation by benzylation after reduction. In other words, it would be necessary to visualize some grouping in the hormones which would be capable of being reduced by cysteine at a neutral pH at room temperature and which, only after reduction, would be benzylated under the conditions used. No grouping other than the disulfide could be found to fit these requirements. Consequently, du Vigneaud felt that there is reason to believe that a disulfide does exist in the pressor and oxytocic hormones; that reduction of this group is obtained by treatment with cysteine; and that the sulfhydryl forms of these hormones are active.

Partial support for the conclusions of Sealock and du Vigneaud was provided by Freudenberg, Weiss and Biller[31] who found that the oxytocic hormone remained fully active after treatment with sodium amalgam in alkaline solution even though sulfhydryl groups were liberated. These workers also provided corroboration for the destruction of oxytocic activity by H_2O_2, alkali, neutral or alkaline sulfite, neutral or alkaline iodine, catalytic reduction and ultraviolet light. However, acid iodine was found to be without effect. Partial reactivation of a catalytically reduced oxytocic preparation was secured by peroxide oxidation. In those cases in which the treatment caused inactivation of the oxytocic hormone, Freudenberg claimed that the reaction proceded less rapidly than did the inactivation of insulin under similar conditions.

Certain aspects of the foregoing results require further examination. If we accept the view that a disulfide linkage is an integral and essential part of both hormone molecules, the inactivating effects of oxidizing agents such as H_2O_2 and the halogens can be explained entirely on the basis of an oxidative destruction of the disulfide linkage. Oxidation of any other susceptible groups in the molecule can, of course, also occur. If we accept the view that reduction of the disulfide linkage to the sulfhydryl form causes no change in activity, the inactivating effects of a number of the reducing agents previously discussed must be explained on some other basis. Reagents like cyanide or sulfite inactivate the hormone and are also capable of reducing disulfide linkages. Because it is believed that such a reduction alone does not cause inactivation, it must be concluded that the inactivation caused by these reagents is due

[61] **du Vigneaud, V.** Cold Spring Harbor Symposia on Quant. Biol. **VI**: 275. 1938.

to their simultaneous reduction of, or reaction with, additional groups in the hormone molecules. This explanation is all the more probable since it is obvious that the disulfide linkage cannot be the only group necessary for the activity of the hormone. SO_2, HCN and HNO_2, which exert no effect upon the disulfide linkage, can be considered to cause their inactivating effects by action upon these unknown groups. Though our knowledge concerning the amino acids probably present in the active principles and the behavior of the hormones to certain chemical treatments provide clues for future investigation, it is as yet much too inadequate to enable us to decide which ones of the several possible groups are involved in these inactivation reactions.

Electrophoresis Experiments

Investigation of the electrophoretic behavior of the pressor and oxytocic principles has yielded some very useful information concerning the electrochemical nature of these hormones. It will be recalled that much of the early chemical work indicated that both principles were predominantly basic in character. In fact Kamm and his associates[3] were led to conclude from their purification studies that both active principles "appear to be basic in character," and accordingly named their isolated products, alpha and beta hypophamine, to signify that they might be amines derived from the hypophysis. The electrodialysis studies of Freeman, Gulland and Randall,[65] and later those of Das, Ghosh and Guha[66] upon purified oxytocic preparations did little to change this view. These investigators found that although migration of the hormone toward the cathode was obtained readily under certain conditions, migration toward the anode did not occur. It was concluded, therefore, that the oxytocic principle was either a base or was adsorbed on basic material.

An observation made in the Cornell laboratory in the course of studies on the purification of the posterior lobe principles by electrophoresis strongly indicated that the hormones are not bases but amphoteric molecules.[5] It was observed during electrophoresis that neither principle was able to enter a compartment where the pH was maintained at 12. Accordingly, in cooperation with Dr. Mildred Cohn,[11] a detailed study of the migration of the pressor principle over a wide pH range was carried out in an apparatus designed for this purpose and in the apparatus of Tiselius. The direction and extent of migration of pressor activity after suitable periods of electrophoresis was determined by assay and the net mobility was calculated. The results plotted in FIGURE 2 show

[65] **Freeman, M., Gulland, J. M. & Randall, S. S.** Biochem. Jour. **29**: 2211. 1935.
[66] **Das, N., Ghosh, B. N. & Guha, B. C.** Zeit. physiol. Chem. **238**: 131. 1936.

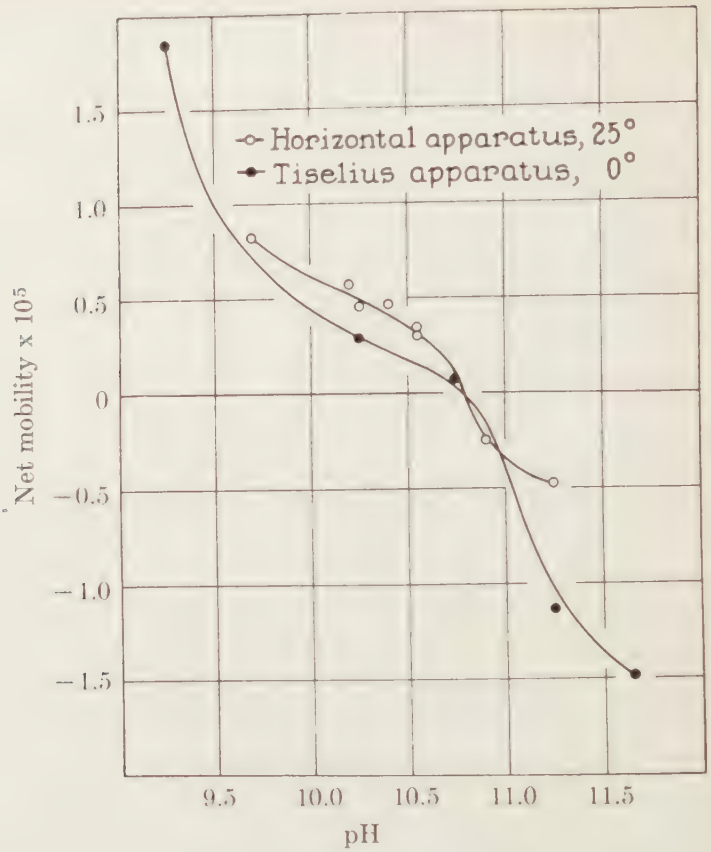

FIGURE 2.

that the pressor principle is definitely amphoteric with an isoelectric point at about pH 10.8 in buffers of 0.02 ionic strength. A preliminary investigation of highly purified oxytocic preparations indicates that this principle is also amphoteric and that it has an isoelectric point in the region of pH 8.5.

It should be mentioned here that in our procedure for the purification of the pressor hormone (TABLE 3), advantage was taken of its high isoelectric point to prevent destruction of activity by cathodic reduction during electrophoresis. The negative electrode was placed in a cell having a pH of 12. Since in migrating toward the cathode the amphoteric principles were unable to enter a solution having a pH above their isoelectric points, electrode reactions were eliminated.

THE UNITARY HORMONE CONCEPT

(ISOLATION OF AN ACTIVE PROTEIN FROM THE POSTERIOR LOBE)

As stated earlier, Abel and his group believed that the pharmacological activities of the posterior lobe were due to a single, large "mother-molecule." This view was maintained even after the experiments of Dudley and Kamm had shown that separate pressor and oxytocic preparations could be obtained from posterior lobe extracts by suitable fractionation procedures. Abel insisted that in such separations the treatments used were sufficiently drastic to cleave the "mother-molecule" producing several small active fractions. The definition of the unitary theory on this basis placed its proponents in a very difficult position because the proof of such an hypothesis rested upon the successful isolation of the supposed "mother-molecule," the demonstration that it possessed all of the pharmacological properties of the posterior lobe, and that it could be split by the suspected treatments to give separate pressor and oxytocic principles. Professor Abel was unable to isolate the hypothetical "mother-molecule" and based his convictions chiefly on his ability to obtain purified preparations in which the ratio between the pressor, oxytocic and melanosome-dispersing activities was the same as that found in untreated gland extracts. But, when it became evident that melanosome-dispersing activity was not a property of the posterior lobe, the basis for Abel's argument became less secure. Valid evidence was also advanced to show that many of Abel's preparations were not the partially purified "mother-molecule" he suspected but mixtures of the separate principles since the activity ratio was occasionally not identical with that of untreated extracts. In some instances, however, the ratio between the various activities accidentally coincided with that found in the gland. Nevertheless, it was realized that Abel's contentions regarding the possibility of a "mother-molecule" could by no means be disregarded.

In 1931 MacArthur[67] sketchily outlined a process for obtaining an active preparation from the posterior lobe which seemed to fit some of the requirements of the hypothetical "mother-molecule," but too few details were given to permit a definite conclusion. The work was never confirmed or extended. The preparation was obtained by acetone and ether precipitation from a 70 per cent methanol (containing 5 per cent acetic acid) extract of dessicated fresh glands. MacArthur's product was a sparingly water soluble substance, possessing both pressor and oxytocic activity and having an isoelectric point at about pH 5.0. Ac-

[67] **MacArthur, C. G.** Science **73**: 448. 1931.

tivity was destroyed by acid hydrolysis and by trypsin. It was unstable in weak alkali and contained labile sulfur.

At this stage in the development of the posterior lobe problem new methods of approach were desired to throw additional light upon the unitary and multiple hormone theories. Electrophoresis seemed to provide such a method.[9] The apparatus used consisted essentially of a series of beakers filled with water and joined by means of inverted U-shaped siphons. Electrodes were placed in the end cells and the pituitary material was dissolved in one of the cells. After a suitable period of electrophoresis the contents of each cell were assayed for pressor and oxytocic activity. When slightly acid solutions of partially purified posterior lobe preparations containing both principles were subjected to electrophoresis in a 19 cell train, it was found that both principles migrated toward the cathode and that the rate of migration of pressor activity was approximately six times that of oxytocic activity. Since the two activities migrated at different rates it was concluded that they were physiological manifestations of different chemical entities in the preparations electrolyzed, a fact which was in agreement with the preponderance of evidence then available.

By means of the same technique it was possible to study also the electrophoretic behavior of the activities contained in the mechanically expressed juice of fresh, untreated glands.[10] Posterior lobes were removed from steers within a few minutes after the animals were killed and were immediately frozen in a tube immersed in dry ice. The glands were thawed, ground with sand and a little water, and the juice was separated by application of high pressure. The juice (pH 6.0, 560 pressor and 420 oxytocic units) was placed in the center cell of a 5-cell setup and electrophoresis was carried out for 17 hours. The time elapsing from the removal of the glands to the end of the electrophoresis was 23 hours, during which time the temperature of the glands and juice was never above 10° C. The results which are given in TABLE 7 show that pressor activity traveled at a faster rate than oxytocic activity, demonstrating that these activities in the simple press juice are attributable to different chemical entities.

We believe that this conclusion was justified since the material studied was subjected to no chemical treatments and since the possibility of preferential destruction of one activity was eliminated by quantitative recovery of both activities. It was realized, however, that these results could not be taken to indicate that the same situation necessarily existed in the gland itself. The elimination of such a possibility was beyond the scope of this approach.

Table 7
Distribution of Activity After Electrophoresis of Posterior Lobe Press Juice

Cell number	Pressor units	Oxytocic units	pH at end of experiment
1 (cathode)	27	3	10.2
2	432	240	6.2
3 (starting)	96	170	4.0
4	3	9	3.0
5 (anode)	0	0	2.3
	558	422	

It is only quite recently that evidence favorable to the unitary hypothesis has been advanced. In 1940 Rosenfeld[39] compared the behavior in the ultracentrifuge of purified pressor and oxytocic preparations with that of untreated press juice from posterior lobes. The purified pressor and oxytocic preparations showed very little tendency to sediment even after almost 6 hours at 61,000 r.p.m. Rosenfeld concluded that the two activities present in such preparations could therefore be attributed to small molecules which might well be of the order of 600 molecular weight, the value estimated by Kamm.[40] On the other hand, both activities sedimented rapidly and at approximately the same rates when press juice was centrifuged. It was concluded that the two activities present in the press juice resided either in a single large molecule (the unitary principle of Abel), or in two separate large molecules having about the same sedimentation rates. The molecular size of the native posterior lobe hormone was provisionally placed at 20,000 to 30,000. To indicate the effects of acid treatment at elevated temperature on the lability of the hormone molecule, a sample of the press juice was mixed with an equal volume of 0.5 per cent acetic acid, brought quickly to boiling, cooled and filtered. Ultracentrifugation of the filtrate showed the presence of both slowly and rapidly sedimenting active particles. Rosenfeld concluded from this result that a portion of the native hormone had been split by the treatment into small, active fragments.

In the foregoing experiment, as in many experiments where claims for the demonstration of a large active molecule have been made, the possibility of adsorption of smaller, extremely active substances upon a biologically inert substance cannot be ignored. In Rosenfeld's work the possibility that adsorption could account for his results with press juice has not been entirely eliminated. In this respect experiments in which known amounts of activity in the form of low molecular weight preparations are added to the press juice before centrifuging would be

TABLE 8

PURIFICATION PROCEDURE OF VAN DYKE AND HIS COLLABORATORS[68]

Fraction	Weight	Procedure	Potency, units per mg.		Total activity	
			Pressor	Oxytocic	Pressor	Oxytocic
					Per cent	*Per cent*
A	1 kg.	Fresh posterior lobes Ground and suspended in 0.01N H_2SO_4, pH 4.25	0.2	0.2	100	100
B	—	Supernatant from centrifuging pH adjusted to 3.9; 80 gm. NaCl added per liter.	—	—	75–80	75–80
C	—	Precipitate Dialized Cl^- free; 10 gm. NaCl added per liter; pH 3.5	—	—	50	50
D	—	Supernatant from centrifuging Equal vol. M/1 acetate buffer, pH 3.9 added; 20 gm. NaCl added per liter.	—	—	—	—
E	—	Supernatant from centrifuging and washing. 40 gm. NaCl added per liter.	—	—	—	—
F	—	Precipitate Dissolved in minimum volume of water; equal vol. M/1 acetate buffer added; 6.5 gm. NaCl added per 100 cc.	—	—	—	—
G	—	Precipitate Previous step repeated until dissolved N is constant (0.100 mg. per cc. at 25°C).	—	—	—	—
H (Active Protein)	700 mg.	Precipitate	17	17	6	6

of value. If the native protein present in the press juice exerts no adsorptive action, this added activity should fail to sediment appreciably. Rosenfeld's experiments do not take into account the possibility that the

TABLE 9
PROPERTIES OF THE POSTERIOR LOBE PROTEIN

Activity, units per mg.	
Pressor	16.6
Oxytocic	16.6
Antidiuretic	16.4
Elementary analysis, per cent	
Carbon	48.64
Hydrogen	6.63
Nitrogen	16.32
Amino nitrogen	0.054
Phosphorus	0.027
Sulfur	4.89
Chlorine	0.02
Ash	0.58
Oxygen (by difference)	22.89
Sulfur distribution	
Cysteine	0
Cystine	4.3
Methionine	?
Sulfate	0.1–0.4
Molecular weight (ultracentrifuge)	30,000
Isoelectric point (electrophoresis)	4.8

brief heat treatment of the press juice might alter the adsorptive properties of the native protein through partial denaturation, thereby liberating a portion of the adsorbed activity.

The current work of van Dyke and his associates places the concept of a unitary hormone in the forefront of present biochemical research on the pituitary problem. Since the evidence obtained by these investigators is of great significance in our evaluation of the multiple and unitary hormone theories, a detailed description of their results is in order.[68]

The purification procedure used by van Dyke and his collaborators is presented schematically in TABLE 8. From 1 kg. of posterior lobes 700 mg. of the purified product are obtained; the product is an amorphous protein. It has a pressor, oxytocic and antidiuretic activity of 17 units per mg. and contains, therefore, about 6 per cent of the activity present in the original material. It is practically devoid of melanosome-dispersing activity.

The composition and properties of the isolated protein are tabulated in TABLE 9. The molecular weight, calculated from the amino nitrogen value, assuming one amino group per molecule, is 26,000. This agrees fairly well with the figure calculated from ultracentrifuge data. The

[68] **Van Dyke, H. B., Chow, B. F., Greep, R. O., & Rothen, A.** Jour. Pharmacol. **74**: 190. 1942.

cystine sulfur value is very high and corresponds to a content of about 16 per cent cystine or 20 cystine molecules in a protein of 30,000 molecular weight.

The active protein is only 25 per cent inactivated by the action of crystalline pepsin at pH 3 and 37° C. for 6 days. Under these conditions 36 per cent of the protein is digested as calculated from the amino nitrogen increase. In similar experiments with trypsin and chymotrypsin (pH 7.6 for 43 hours) the activity is almost completely destroyed. With trypsin 70 per cent of the protein is digested; with chymotrypsin, 95 per cent is digested. Only oxytocic activity was tested in these experiments.

The assay of the protein by various procedures is given in TABLE 10.

TABLE 10

ASSAY OF THE PROTEIN FROM THE POSTERIOR LOBE

Method of assay	Average activity.* Units per mg. protein	Standard error*
Isolated guinea pig uterus	17.5	0.9
Blood pressure of fowl	15.7	0.6
Blood pressure of cat	15.7	—
Blood pressure of dog	17.5	0.6
Diuresis inhibition in rat	16.4	—
Melanosome dispersion in frog	0.008	

*Van Dyke and coworkers express their potencies in micrograms of nitrogen equivalent to 1 U.S.P. unit. To simplify comparison with previous work, these figures have been recalculated on the basis of units per mg. of protein.

It will be noted that the various activities are present in equal amounts in the purified product, a condition which is one of the requirements of the unitary hormone. Consequently, the isolated protein was carefully examined to determine its homogeneity and to demonstrate as convincingly as possible whether or not the pressor, oxytocic and antidiuretic activities are integral parts of the protein molecule, since it was realized by van Dyke and his associates that the purity and inherent activity of the isolated protein must be established beyond all doubt if it is to be considered the "mother-molecule" and probable precursor of the separate posterior lobe hormones.

The group of workers at the Squibb Institute has gone to great lengths to provide the required proof. Their results appear to indicate that: (1) The protein possesses a constant ratio of pressor, oxytocic and antidiuretic activities (1:1:1). (2) The ratio of activities in the protein is identical to that existing in the untreated gland. (3) The protein exhibits a solubility curve typical of a pure substance. (4) The protein

sediments in the ultracentrifuge as a pure protein. (5) Assay of various fractions from solubility, electrophoresis and ultracentrifuge tests gives no indication of the presence of components of greater or lesser potency than the protein itself. Though van Dyke and his associates believe that the results of these experiments are strongly in favor of the homogeneity of the product and the presence of the activities as integral parts of the protein, they nevertheless suggest that the results obtained so far are open to two interpretations: (1) The protein, although pure to the extent that present physicochemical methods permit such a conclusion, is pharmacologically active because of the adsorption of the highly active separate principles. (2) The protein, in part composed of active principles which can be separated from it, is elaborated and stored by the *pars neuralis*.

It is apparent that the first of the above alternatives must be ruled out completely before the second explanation can be accepted. Consequently, it may be profitable to ascertain what has been established by the researches of van Dyke and coworkers and to suggest any additional experiments which might aid in the eventual solution of this problem.

Solubility Data

Constant solubility (0.1 mg. nitrogen per ml.) is obtained with the protein in a solvent consisting of 0.5 M acetate buffer, pH 3.90, to which 6.5 gm. of NaCl are added for each 100 cc. In the same solvent constant solubility as low as 0.08 mg. nitrogen per ml. was also found with some batches of the isolated protein. No evidence for the presence of more than one component could be found in the solubility curve either before saturation with the active product or after 20 times the saturating concentration was in suspension. Over the range of the entire solubility curve the dissolved pressor and oxytocic activities were found to be equal and constant. The pressor and oxytocic potency of a sample of the pure protein was found to be unchanged after it was washed three times with the solvent used in the solubility tests, even though 54 per cent of the total activity was removed in the process. Furthermore, the supernatant fluid from a suspension of the protein in the same solvent, under conditions in which the solid phase was present far in excess of the saturation concentrations, showed the same pressor and oxytocic potency as the protein itself.

Solubility tests have been made only in a solvent of pH 3.90. As this solvent is identical in pH and composition with that used in attaining constant solubility in the final steps of the isolation procedure (see TABLE

8), the subsequent determination of solubility under the same conditions would be expected to furnish no additional information regarding purity. In fact, use of the same solvent in the isolation and in the purity tests would maintain any conditions responsible for the possible association of adsorbent and adsorbate if such should be the case. In this connection it may be significant that electrical inhomogeneity was found in electrophoresis experiments in which the pH of the medium was varied between 3.4 and 6.4, whereas no evidence of inhomogeneity was observed in solubility and ultracentrifuge experiments when the pH was allowed to vary only from pH 3.3 to 3.9. When sufficient amounts of the protein are available to permit such experiments, solubility curves, together with assays for each of the activities at different points in the solubility curve should obviously be carried out at several pH values, and, if possible, in solvents of different composition. If conditions could be found in which the active material is dissociated from the protein, it should be possible to isolate the inactive protein. Suitable amounts of the separated principles could then be added to the inactive protein and experiments could be carried out to determine whether or not a product of constant solubility and activity is again obtained under the conditions used in the original isolation procedure. These data should provide information as to whether the protein is a pure, active substance which is fragmented by the treatments employed, or a protein whose activity is simply adsorbed or eluted depending upon the conditions of the solvent. If dissociation of the protein and activity cannot be accomplished under any of the conditions employed, it would be desirable to study the solubility curve and activity at different points on the curve, after adding known amounts of the separated active principles to the active protein. If activity is actually adsorbed then it might be possible, for instance, to isolate a product having a pressor:oxytocic ratio greater than unity by precipitating the protein in a solution which has been enriched with respect to the pressor principle through the addition of known amounts of a purified pressor preparation.

Ultracentrifuge Data

In the ultracentrifuge, in the pH range 3.30 to 3.84, the isolated product appears to be a single, homogeneous protein with a sedimentation constant of 2.61 to 2.80 S*. From this figure, and an assumed specific volume of 0.749, a molecular weight of approximately 30,000 is calculated. Homogeneity was shown by the symmetry of the sedimentation curves and by the absence of significant displacement of the base line.

* All sedimentation constants are expressed in Svedberg units, denoted S, and equal to 10^{-13} sec.

The distribution of activity between the sedimented protein fraction and the supernatant fluid was determined after ultracentrifuging. Almost 100 per cent of the pressor and oxytocic activity was found to sediment with the protein. The authors concluded from these data that both pressor and oxytocic activities were associated with the protein and that there is no evidence favoring the presence of additional small, nonprotein components with high biological activity.

For the reasons presented in the discussion of the solubility data, ultracentrifuge experiments with the active protein should be tried at widely different pH values. Similarly, the behavior of the active protein in the presence of known amounts of the separate principles should also be investigated in the ultracentrifuge under the conditions reported for the protein alone. In the latter experiments it should be possible to demonstrate whether or not the added, low molecular weight principles sediment with the protein under these conditions.

Electrophoresis Data

The isoelectric point of the protein was estimated to be pH 4.8 by determining the electrophoretic mobility over a pH range from 3.4 to 6.2. At pH 3.41 to 3.47 the electrophoretic patterns showed the presence of one main component, but a second component, present in very small amount, also appeared. At all other pH values studied, electrical inhomogeneity was found but no second component could be discerned.

After electrophoresis of solutions of the protein at pH 3.4 to 3.5 the potencies of the different fractions (main component, "portein-free solution," and the minor component) were indistinguishable on the basis of nitrogen. Accordingly, it was suggested that the minor component either derived from or was closely related to the main protein component.

Interpretation of these results is difficult. Electrophoretic patterns obtained with the isolated protein give definite evidence of inhomogeneity, yet assays performed upon various fractions of the electrophoresis cell apparently fail to reveal the presence of substances of greater or lesser potency than the protein. Inasmuch as electrical inhomogeneity exists it is rather surprising that all of the components of the mixture possess the same pharmacological potency. Experiments should be devised in which separation of the components is accomplished under conditions such that sufficient active material is present in each of the fractions to afford complete and accurate assays for all of the activities. In this connection it will be recalled that previous electrophoresis experiments on untreated posterior lobe press juice,[19] performed under conditions which differed from those of van Dyke and his associates,

gave evidence in favor of the presence of separate pressor and oxytocic hormones. In the electrophoresis of the active protein, therefore, there is a possibility that the electrical inhomogeneity observed at certain pH values may be an indication of the separation of protein and adsorbed active principles.

The recovery of all activities should be investigated in electrophoresis experiments to show that preferential destruction of certain activities has not occurred, and to show that appreciable amounts of any rapidly moving pressor and oxytocic hormones present have not migrated beyond the limits of the electrophoresis cell. Experiments in which alternating current of the same potential used in the electrophoresis is employed might be used to ascertain the effect of the current alone upon the active protein. The homogeneity of material treated in this manner could then be studied by means of solubility and ultracentrifuge tests.

Since from the standpoint of the unitary hypothesis so much depends upon the constancy of the ratio between the activities, it must be emphasized that assays for all of the activities should be carried out upon the various fractions obtained during solubility, electrophoresis and ultracentrifuge experiments, and in any other experiments in which the separation of activities is a possibility.

Action of Cysteine Upon the Active Protein

A significant difference between the separate pressor and oxytocic principles and the active protein is found in the behavior of these substances toward the reducing action of cysteine and thioglycollic acid. Sealock and du Vigneaud[9] found that cysteine reduction of the pressor and oxytocic hormones caused no loss of pharmacological activity. On the other hand, van Dyke and coworkers find that treatment of the protein with thioglycollic acid at pH 7.5 completely destroys both pressor and oxytocic activity in 2 to 5 minutes. With cysteine instead of thioglycollic acid, activity is destroyed but at a slightly slower rate. A 44 per cent loss of activity was found after 2 minutes and after 5 minutes approximately 80 per cent loss occurred.

The observations that cysteine and thioglycollic acid destroy the activity of the protein but cysteine causes no inactivation of the separate principles, may seem to indicate rather strongly that the active protein is not merely an inactive substance with adsorbed active principles. The importance of these results warrants careful repetition under rigorously controlled conditions. It would be desirable to know whether the results of Sealock and du Vigneaud can be confirmed when the separate principles are subjected to cysteine reduction under the conditions used

by van Dyke and associates. If confirmation of the results with the separate principles is secured, while treatment of the active protein under exactly the same conditions still causes inactivation, it will be necessary to repeat the procedure on a mixture of the protein and the separate principles to determine whether the presence of protein during the reduction may be responsible for the difference between the behavior of the two preparations. It should be pointed out that the results as reported by Sealock and du Vigneaud and by van Dyke and associates are not necessarily incompatible. Reduction of the purified individual principles may be quite different from the reduction of the protein complex reported by van Dyke and his collaborators.

SUMMARY

The present status of the posterior pituitary hormone problem may be briefly summarized as follows. Separate pressor and oxytocic hormones can be isolated from the posterior pituitary lobe. These hormones have not yet been obtained in the pure state but all evidence points to the fact that the highly potent pressor and oxytocic preparations now available represent fairly pure preparations of separate molecules which may be similar chemically.

A protein which possesses pressor, oxytocic and antidiuretic activity has also been isolated from the posterior lobe. Moreover, evidence has been presented which indicates that this protein is pure and that the various activities are inherent properties of the molecule. Although this evidence is in favor of the tentative conclusions reached, it is believed that the question should be investigated further before the protein can be accepted unqualifiedly as a hormone of the posterior lobe possessing multiple activities.

THE CHEMISTRY OF THYLAKENTRIN, THE FOLLICLE-STIMULATING HORMONE OF THE ANTERIOR PITUITARY

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INTRODUCTION

Significant progress has been made in the chemical characterization of three anterior pituitary hormones which contribute to the regulation of the gonads. They are: metakentrin,[1-4] a hormone which stimulates the interstitial cells of the ovaries or testes and causes the formation of *corpora lutea* from preformed graafian follicles; thylakentrin,[5-7] a gametogenic hormone which causes the growth of graafian follicles preparatory to the release of ova from the female gonad and stimulates the sperm-forming tissue of the testes; and lactogenic hormone,[8,9] recently shown to have an important role in bringing about *corpus luteum* function[10-12] in hypophysectomized rats. Other gonadotrophic principles such as the antagonist[13] and synergist[14] have been postulated. The present data[15-18] favor the view, however, that synergism (or augmentation) is due to the combined action of thylakentrin and metakentrin on the ovary and that the antagonist[19-21] is identical with metakentrin. Since the chemistry of metakentrin (ICSH, LH), and lactogenic hormone, will be presented in other communications, these hormones will

[1] **Fevold, H. L., Lee, M., Hisaw, F. L. & Cohn, E. J.** Endocrinology **26**: 999. 1940.
[2] **Li, C. H., Simpson, M. E., & Evans, H. M.** Endocrinology **27**: 803. 1940.
[3] **Shedlovsky, T., Rothen, A., Greep, R. O., van Dyke, H. B., & Chow, B. F.** Science **92**: 178. 1940.
[4] **Cohn, H. C. & van Dyke, H. B.** Science **93**: 61. 1941.
[5] **Fraenkel-Conrat, H., Simpson, M. E. & Evans, H. M.** Annales de la Facultad de Medicina, Montevideo. XXV. 1940.
[6] **Greep, R. O., van Dyke, H. B., & Chow, B. F.** Jour. Biol. Chem. **133**: 289. 1940.
[7] **McCullagh, D. R. & Bowman, W. E.** Endocrinology **27**: 525. 1940.
[8] **White, A., Catchpole, H. R. & Long, C. N. H.** Science **36**: 82. 1937.
[9] **Li, C. H., Lyons, W. R. & Evans, H. M.** Jour. Gen. Physiol. **24**: 303. 1941.
[10] **Evans, H. M., Simpson, M. E. & Turpeinen, K.** Anat. Rec. Suppl. 3. **70**: 26. 1938.
[11] **Evans, H. M., Simpson, M. E., Lyons, W. R. & Turpeinen, K.** Endocrinology **28**: 933. 1941.
[12] **Evans, H. M., Simpson, M. E. & Lyons, W. R.** Proc. Soc. Exper. Biol. and Med. **46**: 586. 1941.
[13] **Evans, H. M., Korpi, K., Pencharz, R. F. & Simpson, M. E.** Univ. of Calif. Publ. in Anatomy **1**: 237. 1936.
[14] **Evans, H. M., Pencharz, R. F. & Simpson, M. E.** Endocrinology **18**: 601. 1934.
[15] **Fevold, H. L. & Hisaw, F. L.** Am. Jour. Physiol. **109**: 655. 1934.
[16] **Jensen, H., Simpson, M. E., Tolksdorf, S. & Evans, H. M.** Endocrinology **25**: 67. 1939.
[17] **Fraenkel-Conrat, H., Li, C. H., Simpson, M. E. & Evans, H. M.** Endocrinology **27**: 793. 1940.
[18] **Greep, R. O., van Dyke, H. B., & Chow, B. F.** Am. Jour. Physiol. **133**: 303. 1941.
[19] **Fevold, H. L. & Fiske, V. M.** Endocrinology **24**: 823. 1939.
[20] **Jensen, H., Simpson, M. E., Tolksdorf, S. & Evans, H. M.** Endocrinology **25**: 57. 1939.
[21] **Greep, R. O., van Dyke, H. B., & Chow, B. F.** Am. Jour. Physiol. **133**: 303. 1941.

not be considered in this report. The present discussion will therefore be limited chiefly to the follicle-stimulating hormone, thylakentrin.

METHODS OF ASSAY

Ovaries. Thylakentrin induces the growth of a large number of graafian follicles resulting in gross enlargement of the ovaries. This effect, usually measured by changes in weight, less commonly by histological means, has formed the principal basis of assay. No other single gonadotrophic agent is known to increase significantly the weight of the ovary.

In order to avoid the complication caused by the secretion of hormone by the test animals' own pituitaries, it is necessary to use animals in which this gland has been surgically removed (hypophysectomy). Recent work[20,21] dictates that not only the ovaries of such animals treated with thylakentrin must show the absence of *corpora lutea* to indicate a purely follicle-stimulating effect, but also that the interstitial tissue must be shown to be in an atrophic condition.

Uterus. Estrogenic development of the female reproductive tract is a constant feature of the sexual precocity induced in immature animals by certain gonad-stimulating preparations. Some workers[22] have utilized the increase in the weight of the uterus as an indirect method of measuring ovarian stimulation presumed to be follicle-stimulating in effect. While this method will undoubtedly yield quantitative data, the basic assumption that the response is a measure of any specific gonadotrophic effect can be seriously questioned. The mechanism of estrogen secretion by the ovary is itself an unsolved problem. As we shall see later, thylakentrin alone appears not to be able to cause estrogen secretion; therefore, follicle growth can be obtained independent of any uterine growth.

Vagina. Some authors [23] have recommended the production of vaginal oestrus as a basis for the assay of thylakentrin. The objections mentioned in the discussion of assay by determining the extent of uterine hypertrophy apply with equal force to vaginal changes which also appear not to afford a reliable method of assay.

Ovulation in the Rabbit. This method of assay is not specific but might have quantitative value provided that biological purity had been demonstrated.

Testes. The gonads of hypophysectomized male rats show an increase in weight due solely to stimulation of the sperm-forming tissue.

[22] **Levin, T. & Tyndale, H. H.** Endocrinology **21**: 619. 1937.
[23] **Witschi, E.** Endocrinology **27**: 487. 1940.

This reaction is not accompanied by any testicular secretion of androgens; hence the secondary sexual organs such as the prostate and seminal vesicles are not benefited. The weight of the testes bears a quantitative relationship to the dose of thylakentrin administered within a restricted range. This method[24] can be used only when metakentrin, which also causes an increase in the weight of the testes (presumably by a secondary reaction to the androgen produced), is absent.

SOURCES

The gonadotrophic hormone, thylakentrin, may be obtained from pituitary glands or from the urine of normal, menopausal or gonadectomized human beings. This hormone in the urine can be concentrated by precipitation with tannic acid,[25] [26] alcohol[27] or by other means without much loss of activity. By further fractionation of the hormone "tannate," a preparation effective in producing follicle stimulation, but essentially devoid of luteinizing ability, can be obtained. So far little chemical data have been accumulated on the follicle-stimulating hormone from urine.

Preparation of the Thylakentrin from Pituitary Glands

Both human and horse pituitary glands are relatively rich in thylakentrin but poor in metakentrin. It would appear that from such raw material, potent thylakentrin fractions free from metakentrin are likely to be obtained. But the practical difficulty of securing adequate supplies of human or equine glands has usually forced investigators to turn to the less suitable but more plentiful sheep and hog glands.

Ever since Fevold, Hisaw, and Leonard[28] asserted that they were able to separate the luteinizing from the follicle-stimulating hormone by chemical fractionation, many investigators have attempted to reach the same goal by improved methods. As a whole, the earlier workers used acetone-dried pituitary powder as the starting material. As knowledge of the chemistry of proteins became more general, however, fresh pituitary glands instead of dried powders were preferred, for acetone is known to denature and alter proteins.

Common procedures used to effect the separation of the two gonadotrophic hormones by different workers may be summarized as follows:

1. Evans[29] and his collaborators utilized the greater solubility of

[24] **Greep, R. O., van Dyke, H. B. & Chow, B. F.** Anat. Rec. **78**: 88. 1940.
[25] **Hellbaum, A. A., Fevold, H. L. & Hisaw, F. L.** Proc. Soc. Exp. Med. **32**: 1566. 1934–35.
[26] **Levin, L.** Endocrinology **28**: 378. 1941.
[27] **McCullagh, D. R. & Bowman, W. E.** Endocrinology **27**: 525. 1940.
[28] **Fevold, H. L., Hisaw, F. L., & Leonard, S. L.** Am. Jour. Physiol. **97**: 291. 1931.
[29] **Evans, H. M., Korpi, K., Pencharz, R. F. & Simpson, M. E.** Univ. of Calif. Publ. in Anatomy **1**: 237. 1936.

thylakentrin in ammonium sulfate solution. In his recent paper with Fraenkel-Conrat,[30] an improved scheme has been published. Other investigators[31,32] also utilized the same technique to effect a separation.

2. A method of Fevold, like that of Evans, depended on the greater solubility of thylakentrin in a certain concentration of ammonium sulfate or acetone. He made use of his discovery that metakentrin shows a minimum solubility at pH 4.2.

3. Wallen-Lawrence[33] separated metakentrin from thylakentrin by precipitation with alcohol at −6° C. The precipitate at 40 per cent alcohol was predominantly luteinizing, while that at 55 per cent alcohol was follicle-stimulating.

4. In our laboratory,[6] we found that hog metakentrin is extremely insoluble in a solvent consisting of M/4 acetate buffer at pH 4.4 and 20.5 per cent Na_2SO_4, whereas thylakentrin is very soluble in such a solvent.

BIOLOGICAL ACTIVITY OF FOLLICLE-STIMULATING EXTRACTS

It is impossible to compare quantitatively the biological potency of the products isolated in the different laboratories because of differences in assay technique. Furthermore, a comparison of potency is no indication of relative chemical purity because the extracts have often been made from glands of different species of animals. But a qualitative difference needs emphasis: the injection, in relatively large doses, of thylakentrin preparations made by workers of other laboratories causes uterine hypertrophy, whereas our purified preparations fail to show any such stimulation. This can be seen from TABLE 1. Although as little as 3 micrograms solid of the Fraenkel-Conrat preparation gave follicular stimulation but no estrous uteri, estrus was definitely produced in 50 per cent of the rats if the dose was increased to 18 micrograms of solid. Similarly, Fevold's preparation of thylakentrin increased uterine weight. Greep, van Dyke and Chow[34] prepared extracts of thylakentrin from hog pituitary glands. Although 62.5 micrograms of their extract stimulated the growth of follicles in hypophysectomized rats, ten times such a dose did not cause estrogen secretion. To test more rigidly the question whether thylakentrin, when essentially free from metakentrin, causes estrogen secretion, these authors injected 1562 micrograms of thylakentrin into hypophysectomized rats over a period of ten days. None of the treated rats was in estrus.

[30] **Fraenkel-Conrat, H., Simpson, M. E. & Evans, H. M.** Annales de la Facultad de Medicina, Montevideo. XXV: 1940.
[31] **Rinderknecht, H. & Williams, P. C.** Jour. Endocrinology **1**: 117. 1939.
[32] **Jensen, H., Tolksdorf, S., & Bamman, F.** Jour. Biol. Chem. **135**: 791. 1940.
[33] **Wallen-Lawrence, Z.** Jour. Pharmacology & Exp. Therap. **51**: 263. 1934.
[34] **Greep, R. O., van Dyke, H. B. & Chow, B. F.** Jour. Biol. Chem. **133**: 289. 1940.

TABLE 1

THE UTERINE RESPONSE OF HYPOPHYSECTOMIZED RATS RECEIVING PURIFIED THYLAKENTRIN (FSH) PREPARED FROM DIFFERENT LABORATORIES.

Method of preparation	Dose in micrograms solid	Ovaries			Uterus
		Number of rats	Weight in mg.	Histology	
Fraenkel-Conrat, Simpson & Evans[a]	0	100	11	O	0*
	3 (1 unit)	9	14	F	0*
	15	8	30	F	1*
	18	8	39	F	4*
Fevold[b]	0	—	6	—	—
	20	—	10	—	0†
	40	—	16	—	0†
	80	—	24	—	50%†
	200	—	31	—	70%†
	500	—	35	—	170%†
Greep, van Dyke & Chow[c]	0	28	8.63	O	0*
	62.5	4	12.47	F	0*
	625.0	5	16.28	F	0*
	1562.0 (10 days)	6	15.1	F	0*
	0 (10 days)	6	5.7	O	0*

* Number of rats with estrous uteri.
† Percentage increase in average uterine weight.
[a] **Fraenkel-Conrat, H. L., Simpson, M. E. & Evans, H. M.** Proc. Soc. Exp. Biol. & Med. **45**: 627. 1940.
[b] **Fevold, H. L.** Endocrinology **28**: 33. 1941.
[c] **Greep, R. O., van Dyke, H. B. & Chow, B. F.** Endocrinology **30**: 635. 1942.

Although Fraenkel-Conrat and Fevold prepared pituitary extracts from sheep glands, and Greep, van Dyke and Chow obtained theirs from hog glands, it seems probable that the question of estrogen secretion of rats receiving thylakentrin extract is more dependent on the degree of contamination by metakentrin than on a difference in the animal source of the extract. The maximum amount of metakentrin present in our thylakentrin preparation as contaminant was determined in two ways:

1. THE PROSTATE METHOD. Greep, van Dyke, and Chow[35] found that as little as 2 micrograms of pure metakentrin nitrogen isolated from hog pituitary glands can bring about a significant increase in the weight of the anterior prostate of hypophysectomized rats. The weight of the anterior prostate was not significantly increased by 360 micrograms of nitrogen of our best preparation of thylakentrin. It might be added also that as little as 2 to 5 micrograms nitrogen of our thylakentrin preparation could bring about a significant increase in the testicular weight. Since the metakentrin nitrogen in the total of 360 micrograms must be

[35] **Greep, R. O., van Dyke, H. B., & Chow, B. F.** Proc. Soc. Exp. Biol. and Med. **46**: 644. 1941.

less than 2 micrograms, the contaminating hormone must be less than 0.5 per cent.

2. The Immunological Method. When rabbits were immunized against pure hog metakentrin, antibodies[36] may be detected in the sera of the immunized animals by the precipitin reaction. Such antisera will react with as little as one microgram of hog metakentrin protein, but will not react with metakentrin from other species of animals or with other pituitary hormones from hog glands. Once the immunological specificity of the anti-hog-metakentrin sera is established it is possible to detect the amount of metakentrin present in various fractions. It was found that 2500 micrograms of our hog thylakentrin extract failed to react with anti-metakentrin rabbit sera. Thus the thylakentrin extract contained less than 0.04 per cent of metakentrin. Tests for other pituitary hormones such as the lactogenic, thyrotrophic, chromatophorotropic, posterior lobe, and adrenotrophic hormones gave uniformly negative results. Therefore, our preparation of thylakentrin may be considered biologically pure in the sense that no other hormones have been found by biological assay to be present in the thylakentrin extract.

PHYSICO-CHEMICAL PROPERTIES

Effect of Enzymes

The action of proteolytic enzymes on pituitary gonadotrophic hormones has been studied by several groups of investigators. Tryptic digestion of pituitary extracts has yielded divergent results.

Chen and van Dyke[37] found that tryptic digestion abolishes most of the luteinizing action of extracts of sheep or horse pituitary, but leaves the follicle-stimulating factor unimpaired. Large doses of the digested extracts were followed by the formation of lutein tissue in the ovaries of hypophysectomized immature rats. Similar results were independently reported by McShan and Meyer[38]. Both groups of authors used normal as well as hypophysectomized rats and studied the microscopic appearance of the ovaries. The destructive effects of crystalline trypsin on thylakentrin were not recognized by McShan and Meyer. In neither set of observations was the extent of digestion determined. The differential lability of thylakentrin and metakentrin to tryptic action was not confirmed by Abramowitz and Hisaw[39]. They found that crystalline trypsin destroys both gonadotrophic hormones at about the same rate. Their conclusions were based on the changes in weight of the ovaries or

[36] **Chow, B. F.** Endocrinology **30**: 657. 1942.
[37] **Chen, G., & Van Dyke, H. B.** Proc. Soc. Exp. Biol. & Med. **40**: 172. 1939.
[38] **McShan, W. H. & Meyer, R. K.** Jour. Biol. Chem. **126**: 361. 1938.
[39] **Abramowitz, A. A. & Hisaw, F. L.** Endocrinology **25**: 633. 1939.

seminal vesicles of normal rats. Lacking histological studies and determinations of extent of digestion, their data do not appear to justify the belief that neither crystalline trypsin nor chymotrypsin destroys luteinizing hormone more rapidly than follicle-stimulating hormone.

Chow, Greep and van Dyke[40] carefully re-examined the effects of digestion by proteolytic enzymes (including crude and crystalline trypsin) on the gonadotrophic activities of anterior pituitary by comparing the actions of digested and incubated control extracts in hypophysectomized immature female and male rats. We found that the percentage of protein digested was often of decisive importance in determining destruction or survival of the hormones. Thus, if the tryptic digestion of the crude hog pituitary extract which contains both metakentrin and thylakentrin had proceeded to a moderate extent (less than 48 per cent), follicular growth was stimulated but the luteinizing action was absent. If digestion was carried to 61 to 75 per cent, both follicle-stimulating and luteinizing activities were abolished. It seemed reasonable to us, therefore, that the discrepancy between the results of Abramowitz and Hisaw, and those of other authors could be explained on the basis of differences in the degree of digestion.

In a recent paper, McShan and Meyer[41] described a reproducible method by which a crude extract, containing both follicle-stimulating and luteinizing activities, can be converted to a product having follicle-stimulating activity only. The degree of digestion was followed by the increase of amino-nitrogen content. Their results, in agreement with ours, showed that although the luteinizing activity can be easily destroyed by tryptic digestion even to a moderate extent, the survival of the follicle-stimulating activity is dependent upon the degree to which the gonadotrophic extracts are digested by trypsin. The same authors further showed that ptyalin or takadiastase destroys the biological activity of thylakentrin. Since these enzymes are assumed to attack carbohydrates but not proteins, it seems reasonable to conclude that carbohydrate also plays an important part in the biological activity. The carbohydrate is not separable from the protein by dialysis or by chemical fractionation, and it is assumed that it is united by chemical bonds. Thus thylakentrin is probably a glycoprotein. The proof of such a hypothesis lies in the eventual isolation of this hormone in a chemically pure form. Because of the importance of the carbohydrate group, attempts have been made to identify[42,43] and to correlate the carbohydrate con-

[40] **Chow, B. F., Greep, R. O. & van Dyke, H. B.** Jour. Endocrinology **1**: 440. 1939.
[41] **McShan, W. H. & Meyer, R. K.** Jour. Biol. Chem. **135**: 473. 1940.
[42] **Fleisher, G., Schwenk, E. & Meyer, K.** Nature **142**: 835. 1938.
[43] **Hartmann, M. & Benz, F.** Nature **142**: 115. 1938.

tent of crude extracts containing the follicle-stimulating hormone with biological activity. Evans[44] and others found that although the thylakentrin fraction of sheep pituitary extracts undoubtedly contained more carbohydrate than any other fraction of pituitary hormones, the determination of carbohydrate alone could not be taken as a measure of its follicle-stimulating potency. Therefore, they searched for a more specific chemical characterization of this gonadotrophic hormone and found that a high glucosamine as well as carbohydrate content are valuable guides in the purification of follicle-stimulating hormone. It must be emphasized here that the analyses were made on extracts of unknown chemical purity.

The Action of Other Chemical Agents on the Follicle-Stimulating Activity of Thylakentrin

EFFECT OF KETENE

Ketene is capable of reacting with all active hydrogens in the protein molecule, namely $-NH_2$, $-SH$, phenolic and alcoholic $-OH$. Among these groups, the amino group is most easily acetylated.

Li, Simpson, and Evans[45] subjected both thylakentrin and metakentrin to ketene treatment at pH 5. They found that acetylation for 5 minutes significantly decreased the potency of metakentrin. In contrast with this, reduction in the activity of thylakentrin did not occur in 5 minutes but did occur after 30 minutes of acetylation. The authors concluded from these experiments that the physiological activity of both thylakentrin and metakentrin is dependent on the free amino groups. They assumed that during acetylation under their experimental conditions (30 minutes at pH 5.7), ketene did not react with the hydroxyl group in the carbohydrate part of the molecule, because Neuberger[46] found ketene unable to acetylate the hydroxyl groups in carbohydrate resulting from the hydrolysis of egg albumin for as long as 18 hours at pH 6.0. They further assumed that phenolic groups were not attacked, because Stern and White[47] have shown that at least 45 minutes are necessary to cause appreciable acetylation of the phenolic groups of insulin. Such an assumption may not be entirely valid, because it is conceivable that the rate of reaction between the ketene and the phenolic groups of different proteins may differ. Such assumptions would not have been necessary if enough material were available for the determination of the ratio of the molar fraction of acetyl groups introduced to that of amino

[44] **Evans, H. M., Fraenkel-Conrat, H., Simpson, M. E. & Li, C. H.** Science **89**: 249. 1939.
[45] **Li, C. H., Simpson, M. E. & Evans, H. M.** Jour. Biol. Chem. **131**: 259. 1939.
[46] **Neuberger, A.** Biochem. Jour. **32**: 1443. 1938.
[47] **Stern, K. G. & White, A.** Jour. Biol. Chem. **122**: 371. 1937–1938.

groups acetylated, and also if no decrease of tyrosine groups was found. The acetylation could not occur on the sulfydryl group because ". . . thio groups have not been found in these substances."

EFFECT OF REDUCING AGENTS

In 1935 Maxwell and Bischoff[48] made some chemical studies on the effect of reducing agents on impure pituitary gonadotrophic hormone. They found no detectable loss of activity upon short exposure of hormone to nascent hydrogen, sulfur dioxide, hydrogen sulfide, ferrous sulfate, or hydrogen cyanide.

Different results were obtained by Fraenkel-Conrat, Simpson, and Evans[49]. They used cysteine, which reduces only the disulfide linkage of the protein, and found that unfractionated gonadotrophic extracts or purified thylakentrin, when treated with cysteine at pH 7.7 for two days, were more than 95 per cent inactivated. They therefore concluded that $-S-S-$ linkages are important to biological activity. McShan and Meyer[41] confirmed the inactivation of thylakentrin by reduction with cysteine. Their preparations, made by the trypsin method, were inactivated by treatment with 6 to 40 times their weight of cysteine at room temperature for 48 hours.

Bischoff[50] reopened the question of the reduction of gonadotrophic hormones. He studied the action of both cysteine and cyanide upon the hypophysial gonadotrophic hormones. He allowed cysteine to react with the hormones for 12 or 48 hours, and cyanide for 1 or 24 hours. His results indicated that increasing amounts of cysteine and extension of the reaction time increased the inactivation, but that the pituitary hormones were able to withstand a short exposure (1 hour) to cyanide in alkaline solution. 24 hours of exposure produced some inactivation.

These three groups of workers confirm one another concerning the reduction of biological activity of thylakentrin when it is exposed to cysteine for a period of 24 hours. But Bischoff disagreed with the hypothesis, suggested by Fraenkel-Conrat and coworkers and endorsed by McShan and Meyer, that $-S-S-$ linkages are an essential part of the active group of this gonadotrophic hormone. He suggested that the inactivation produced by a large excess of reagent and prolonged reaction time might be ascribed to side reactions, but he did not suggest what the side reactions might be.

[48] **Maxwell, L. C., & Bischoff, F.** Jour. Biol. Chem. **112**: 215. 1935-36.
[49] **Fraenkel-Conrat, H., Simpson, M. E. & Evans, H. M.** Jour. Biol. Chem. **130**: 234. 1939.
[50] **Bischoff, F.** Jour. Biol. Chem. **134**: 641. 1940.

Interpretations Suggested by Ultracentrifugation or Electrophoresis

Ultracentrifugation or electrophoretic analysis has been helpful in the isolation of many biologically active proteins. The first attempt to concentrate anterior lobe and pituitary-like hormone in the castrate and menopause urine was made by Severinghaus, Levin and Chiles[51]. They found that the gonadotrophic hormone from the urine could be concentrated by ultracentrifugation at 150,000 to 200,000 g. for 4 to 6 hours, indicating a relatively high molecular weight for the hormone, but there appeared to be no marked purification.

Electrophoretic studies of anterior pituitary proteins have been made by Shipley, Stern and White[52]. The preparations used included crude pituitary gland extracts obtained with dilute alkali, glycerol, or saline. The number of the main boundaries detected during electrophoresis was rather small (2–4), and the bulk of the protein was inert. Therefore, electrophoretic analysis of crude extracts has not given useful information on the electrochemical properties of the thylakentrin.

In collaboration with Doctors Shedlovsky and Rothen of the Rockefeller Institute for Medical Research, we have made a few electrophoretic and ultracentrifugal analyses of our biologically pure thylakentrin extracts. Our results showed definitely that they were electrically inhomogeneous and ultracentrifugally polydisperse. Details of all our experiments will not be given, but one typical electrophoretic separation experiment is worth mentioning. Using an acetate buffer of pH = 5.0 and $\mu = 0.05$, we found that one of the protein components moved anodically, while the remaining components moved cathodically (FIGURE 1). Separation into fractions was made mechanically. The number of components expected in each fraction is given in FIGURE 1 and TABLE 2.

[51] **Severinghaus, A. E., Levin, L. & Chiles, J. A.** Endocrinology **23**: 285. 1938.
[52] **Shipley, R. A., Stern, K. G., & White, A.** Jour. Exp. Med. **69**: 785. 1939.

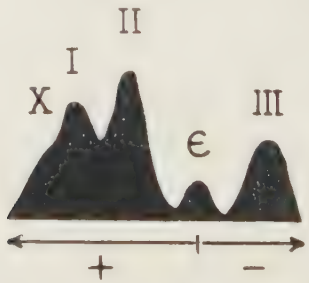

FIGURE 1.

TABLE 2

BIOLOGICAL ASSAY IN HYPOPHYSECTOMIZED RATS OF THE COMPONENTS OBTAINED BY ELECTROPHORETIC SEPARATION OF IMPURE SWINE THYLAKENTRIN.

Fraction number	Components present	Microgram N per dose	Number of rats used	Weight of testes in mg.	Weight of anterior prostate in mg.
5	X + I	10	5	126.4	6.89
6	X + I (II)	10	5	110.4	7.48
7	III	10	3	178.6	6.57
10	III + (II)	10	5	178.6	6.76
Control		0	5	114.7	6.69

All fractions were analyzed for nitrogen and assayed in hypophysectomized male rats. The results of the assay are given in TABLE 2. It can be seen that the biological activity was concentrated in fractions 7 and 10. Fraction 7 contained only component III, but fraction 10 contained component III and a small amount of II. Since component II should also have been present in the inactive fraction 6, it seemed reasonable to conclude that component III was the hormone.

Similar separation experiments were made at pH 4.6. The active component moved as a cation. From the mobilities of the active component at pHs 5.0 and 4.6, we estimated the isoelectric point of hog thylakentrin to be about 4.8.

CONCLUSION AND SUMMARY

In the brief discussion of the chemistry of thylakentrin (FSH) the following points were emphasized:

1. METHODS OF BIOLOGICAL ASSAY. In evaluating different methods, emphasis was placed on the feasibility of quantitative estimation of the thylakentrin present in a given extract. If metakentrin is absent in the test extract, the increase of weight of either ovaries or testes may be used for quantitative assay. If metakentrin is also present, the interpretation of the biological result becomes difficult. Estrogen secretion apparently is not provoked by thylakentrin alone.

2. METHODS OF PURIFICATION. The chief accomplishment in the purification of thylakentrin has been the removal of other pituitary hormone proteins, particularly metakentrin (ICSH). The separation of these two gonadotrophic hormones can be effected by utilizing the relative insolubility of metakentrin in half saturated ammonium sulfate solution, in 40 per cent alcohol at $-6°$C., or in 20.5 per cent sodium sul-

fate buffered with acetate at pH 4.4. Though the purified thylakentrin is not chemically pure, it can be obtained free from other pituitary hormones. Our best preparations of thylakentrin did not stimulate estrogen secretion, even when large doses were given to hypophysectomized rats over a period of ten days. It seems probable that the thylakentrin extracts which cause uterine hypertrophy are contaminated by metakentrin. Estimation of metakentrin (hog) could be made either by the increase of the weight of the ventral prostate of the hypophysectomized rats or by the precipitin test with immune sera of rabbits immunized with pure hog metakentrin.

3. Physico-chemical Properties. Chen and van Dyke, and McShan and Meyer found that tryptic digestion abolishes most of the luteinizing hormone but leaves thylakentrin unimpaired. Abramowitz and Hisaw, however, found that crystalline trypsin destroys both gonadotrophic hormones at about the same rate. We found that these apparently divergent results could be harmonized, if the percentage of protein digested was taken into account. In other words, if the crude hog pituitary extract was digested by trypsin to a moderate extent (less than 48 per cent.), the digest could stimulate follicular growth, but the luteinizing action was absent. If the digestion was carried out further (to 61–75 per cent) both thylakentrin and metakentrin, originally present in the extract, were destroyed.

Thylakentrin can also be inactivated by ptyalin or takadiastase. The assumption is that these enzymes attack only carbohydrate. Since both carbohydrate and protein play an important role in the biological activity, it is further assumed that thylakentrin is a glycoprotein.

The actions of chemical agents like ketene or cysteine have been studied. Acetylation of thylakentrin with ketene for 30 minutes abolished its biological activity. It was assumed that the destruction of the biological activity was due to the acetylation of the amino groups. Cysteine likewise destroyed completely the activity of thylakentrin. Whether this chemical action is one of reduction has not been conclusively proved.

Only a few studies of thylakentrin have been made using either electrophoresis or the ultracentrifuge. The electrophoretic separation of thylakentrin in purified extracts of hog pituitary at different pHs suggested that the isoelectric point of the hormone is about 4.8.

THE LUTEINIZING HORMONE OF THE ANTERIOR LOBE OF THE PITUITARY BODY

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INTRODUCTION

Early indications of the existence of a pituitary luteinizing hormone were obtained by Evans and coworkers.[1] They reported that alkaline extracts of pituitary tissue produced excessive luteinization of the ovaries when injected into rats, while acidified extracts produced follicular growth with less luteinization. They ventured the opinion that the growth hormone might be the factor which produced luteinization. At approximately the same time, Ascheim and Zondek[2,3] reported the discovery of two gonadotrophic hormones in the urine of human beings. One of these stimulated the growth of follicles in the ovaries of rats and the second produced *corpora lutea*. Since it had been shown that the pituitary was essential for gonadal development[4], it was concluded that these substances had their origin in the pituitary. Subsequent work led to a great deal of discussion regarding the unity or duality of the pituitary gonadotropic complex, the question being complicated by the fact that the luteinizing hormone found by Ascheim and Zondek in the urine of pregnancy was not a pituitary hormone and should not have entered into the discussion. The luteinizing hormone of pregnancy urine is of placental origin, is not a pituitary hormone, and will not be considered in this paper.

Chemical fractionation of pituitary gonadotropic extracts resulting in the separation of a luteinizing hormone (LH) from a follicle-stimulating hormone (FSH) was reported in 1931[5]. Following that report, interest in the gonadotropic complex and in the existence of the luteinizing hormone as a separate individual was further stimulated. Further purification of the factor from other pituitary hormones together with physiological characterization of the purified preparations demonstrated that it carried out certain physiological functions which could not be carried out by the follicle-stimulating hormone or by any other pituitary

[1] **Evans, H. M. & Simpson, M. E.** Jour. Am. Med. Assn. **91**: 1337. 1928.
[2] **Ascheim, S. & Zondek, B.** Klin. Wchnschr. **6**: 1322. 1927.
[3] **Zondek, B.** Klin. Wchnschr. **8**: 245. 1930.
[4] **Smith, P. E.** Jour. Am. Med. Assn. **88**: 158. 1927.

hormone. Its place as a separate individual and as an important and indispensible factor in maintaining gonadal function was therefore gradually established.[5–15]

At the present time it appears to be well established that the luteinizing hormone of the pituitary is responsible for and carries out the following physiological functions. Together with the follicle-stimulating hormone it produces estrogen secretion from the ovarian follicles.[16–18] It takes part in the enlargement of the follicles which immediately precedes ovulation and which has been called preovulatory swelling.[19–21] It stimulates the development of luteal tissue and *corpora lutea*, which then secrete progesterone. The luteinizing hormone is not, however, the factor which maintains the *corpora lutea* in a functional, secretory state; a different luteotropic factor from the pituitary carries out that function.[22–23] In the male it acts on the interstitial cells of the testes, stimulating them to secrete the male hormone which then maintains the secondary sex glands and other secondary sex characters. It is therefore an essential substance for reproductive processes, both in male and female—as a luteinizing hormone in one and as an interstitial-cell-stimulating hormone in the other.

In addition to the evidence for the separate existence of the luteinizing hormone obtained by chemical purification and by physiological studies of the purified preparations, important confirmatory evidence for chemical differences in the two gonadotropic hormones has been found in studies of the action of enzymes on pituitary extracts. By subjecting such extracts to controlled digestion with trypsin or chymotrypsin it has been possible to destroy the luteinizing activity of the extracts and still retain the greater part of the follicle-stimulating activity. Conversely, it has been reported that pepsin destroys all of the follicle-stimulating activity with very little destruction of luteinizing activity if the

[5] **Fevold, H. L., Hisaw, F. L. & Leonard, S. L.** Am. Jour. Physiol. **97**: 291. 1931.
[6] **Fevold, H. L., Hisaw, F. L., Hellbaum, A. & Hertz, R.** Am. Jour. Physiol. **104**: 710. 1933.
[7] **Fevold, H. L. & Hisaw, F. L.** Amer. Jour. Physiol. **109**: 655. 1934.
[8] **Wallen-Lawrence, Z.** Jour. Pharmacol. & Exp. Ther. **51**: 263. 1934.
[9] **Evans, H. M., Korpi, K., Simpson, M. E., Pencharz, R. I. & Wonder, D. H.** Univ. of Calif. Pub. in Anat. **1**: 255. 1936.
[10] **Greep, R. O., Fevold, H. L. & Hisaw, F. L.** Anat. Rec. **165**: 261. 1936.
[11] **Greep, R. & Fevold, H. L.** Endocrinology **31**: 611. 1937.
[12] **Fraenkel-Conrat, H., Li, C. H., Simpson, M. E. & Evans, H. M.** Endocrinology **27**: 793. 1940.
[13] **Greep, R. O.** Cold Spring Harbor Symposia Quant. Biol. **5**: 136. 1937.
[14] **Breneman, W. R.** Anat. Rec. **64** (Suppl.): 56. 1935.
[15] **Breneman, W. R.** Anat. Rec. **64**: 211. 1936.
[16] **Fevold, H. L.** Anat. Rec. (Suppl. 2) **73**: 19. 1939.
[17] **Fevold, H. L.** Endocrinology **28**: 33. 1941.
[18] **Shedlovsky, T., Rothen, A., Greep, R. O., van Dyke, H. B. & Chow, B. F.** Science **92**: 188. 1940.
[19] **Foster, M. A. & Hisaw, F. L.** Anat. Rec. **62**: 75. 1935.
[20] **Foster, M. A., Foster, R. C. & Hisaw, F. L.** Endocrinology **21**: 249. 1937.
[21] **Hisaw, F. L., Greep, R. O. & Fevold, H. L.** Anat. Rec. Suppl. **64**: 34. 1935.
[22] **Evans, H. M., Simpson, M. E. & Turpeinen, K.** Anat. Rec. Suppl. **70**: 26. 1938.
[23] **Astwood, E. B.** Endocrinology **28**: 309. 1941.

digestion is not allowed to proceed too far. Ptyalin (saliva) was found to destroy the FSH but not LH.[24–29]

The individuality and specificity of the two gonadotropic hormones is further demonstrated by some immunological experiments which have been reported. It has long been known that antigonadotropic substances are produced in animals when they are injected for long periods of time with gonadotropic extracts. Morever, it has been found that antisera produced against preparations which are rich in LH but poor in FSH will selectively neutralize the LH with very little effect on the FSH when injected together with an extract rich in both.[30]

A recent paper reports some further interesting experiments dealing with the production of antigonadotropic sera.[31] An unfractionated gonadotropic extract was subjected to tryptic digestion which digested the LH but not the FSH. This preparation was then used to produce antisera against FSH. When these antisera were injected with FSH alone, they completely neutralized the FSH effect. When the antisera were injected with an unfractionated extract containing FSH and LH, the FSH action was neutralized but no effect on LH activity was apparent. An antiserum produced by injecting both FSH and LH simultaneously, however, neutralized the effects of both when injected with them.

It is therefore possible to produce antisera which will neutralize either follicle-stimulating hormone or luteinizing hormone or both, depending on whether one or both were present in the extract which was injected to produce the antisera. Thus the two hormones would appear to be separate and distinct proteins.

OCCURRENCE OF THE LUTEINIZING HORMONE

The luteinizing hormone is present in the pituitaries of all animals which have been investigated. There is a species difference in the quantity of luteinizing hormone present, although the information is not too definite due to the fact that different assay methods have been used, and they have usually been carried out in the presence of the follicle-stimulating hormone, which may have some effect on the result. Table 1 lists

24 **McShan, W. H., & Meyer, R. K.** Jour. Biol. Chem. **126**: 361. 1938.
25 **McShan, W. H. & Meyer, R. K.** Proc. Soc. Exp. Biol. Med. **40**: 699. 1939.
26 **McShan, W. H. & Meyer, R. K.** Proc. Soc. Exp. Biol. Med. **40**: 701. 1939.
27 **Chen, G. & van Dyke, H. B.** Proc. Soc. Exp. Biol. Med. **40**: 172. 1939.
28 **Greep, R.** Anat. Rec. (Suppl. 2) **73**: 23. 1939.
29 **Chow, B. F., Greep, R. O. & van Dyke, H. B.** Jour. of Endocrinology **1**: 440. 1939.
30 **Rowlands, I. W.** Jour. of Endocrinology **1**: 172. 1939.
31 **Kupperman, H. S., Meyer, R. K. & McShan, W. H.** Endocrinology **29**: 525. 1941.

TABLE 1

LUTEINIZING HORMONE CONTENT OF THE PITUITARIES OF VARIOUS SPECIES

High	Moderate	Low
Cat	Rat	Beef
Baboon	Armadillo	Whale
Sheep	Guinea Pig	Horse
Opossum	Dog	Human
Cottontail Rabbit	Swine	
	Domestic Rabbit	

the pituitaries of various species in three groups, roughly classifying them with regard to their luteinizing hormone content.[32–35]

Information regarding the occurrence of luteinizing hormone in other tissues is meager. It has been reported to be present in blood but only in small amounts. It is apparently excreted in small amounts, usually together with follicle-stimulating hormone, in the urine of human beings throughout life.[36, 37] The large amount of gonadotropic hormone (Prolan B) excreted in human pregnancy urine, as has already been pointed out, is of placental origin and is physiologically and chemically different from pituitary luteinizing hormone.

The gonadotropic preparations obtained from pregnant mare serum and which produce luteinization may or may not be composed of two substances, one of which is luteinizing hormone. Fractionation of mare serum into two gonadotropic substances has been reported[38, 39] but more recently preparations of very high activity[40] and preparations which are electrophoretically homogeneous[41] have been prepared which are equivalent physiologically to the original crude serum.

There is, however, another piece of evidence which would seem to show that two gonadotropic hormones are present in pregnant mare serum.[31] This comes from immunological data dealing with the production of antisera. It has been reported that antisera produced against sheep pituitary FSH will inhibit the follicle-stimulating activity of pregnant mare serum when injected with it but has no effect on the luteinizing action. If this be substantiated, it would seem to prove the presence of a separate luteinizing protein also in pregnant mare serum. At least

[32] **Hill, R. T.** Jour. Physiol. **83**: 137. 1934.
[33] **Fevold, H. L.** Endocrinology **24**: 435. 1939.
[34] **Witschi, E.** Endocrinology **27**: 487. 1940.
[35] **West, E. & Fevold, H. L.** Proc. Soc. Exp. Biol. Med. **44**: 446. 1940.
[36] **Engle, E. T., Allen, Danforth & Doisy.** "Sex and Internal Secretions." Second Edition. Chapter XVIII. Part II. Williams and Wilkins Co. Baltimore, Md.
[37] **Fevold, H. L. & Fiske, V. M.** Endocrinology **24**: 823. 1939.
[38] **Hellbaum, A. A.** Proc. Am. Physiol. Soc. **119**: 381. 1937.
[39] **Evans, H. M., Korpi, K., Simpson, M. E. & Pencharz, R. I.** Univ. Calif. Pub. in Anat. **1**: 275. 1936.
[40] **Cole, H. H., Pencharz, R. I. & Goss, H.** Endocrinology **27**: 548. 1940.
[41] **Li, C. H., Evans, H. M. & Wonder, D.** Jour. Gen. Physiol. **23**: 733. 1940.

it would prove that follicle stimulation and luteinization are produced by separate prosthetic groups which may or may not be attached to the same protein molecule.

ASSAY METHODS FOR THE LUTEINIZING HORMONE

A number of methods have been and are being used in determining the activity of LH preparations. All of these are biological and depend upon individual physiological properties of the hormone.

The first method used in quantitative work was based on the first known physiological property of the hormone, namely the production of *corpora lutea* in the ovaries of immature rats and the increased weight of the ovaries when LH is injected into immature female rats together with FSH. An amount of FSH is injected over a period of four days which produces approximately 100 per cent increase in weight over that of the ovaries of untreated rats. Various amounts of LH are then injected simultaneously with the same amount of FSH, and the additional increase due to the LH is determined. That amount of LH which produces an additional 100 per cent increase in the weight of the ovaries together with the production of *corpora lutea* is considered a unit.[42] This method has the obvious disadvantage of depending on the interaction of two substances and necessitates the use of FSH which is free of LH. This method can therefore be used only with purified materials.

Other methods, which have been used more often recently, depend on the physiological activity of the luteinizing hormone in the male. LH stimulates the interstitial elements of the testes to secrete androgenic hormone, which then acts on the secondary sex glands such as the prostate and the seminal vesicles. The increase in the weight of any one or of all the secondary sex glands may therefore be used as an indirect measure of the activity of the LH preparation.

The end point which we have used in our laboratory has been the weight increase in the seminal vesicles plus the coagulation gland.[33] Immature male rats, 21 to 22 days old at the beginning of the test, are injected twice daily for 4 days with varying amounts of the material to be tested. The volume of liquid is ¼ cc. per injection. The animals are then killed the morning of the fifth day; the seminal vesicles plus the coagulation gland are dissected and weighed.* From 50 to 100 per cent increase in the weights over those of seminal vesicles of control rats is taken as a unit. The seminal vesicles plus the coagulation glands of untreated rats 25 days old average 10.5 mg. with the limits 8.5 to 12.5

[42] **Fevold, H. L.** Cold Spring Harbor Symposia on Quant. Biol. **5**: 93. 1937.

* The structures removed also include a small portion of the dorsal prostate.

mg. The lowest dosage which increases the weights to 17 or 18 mg. is the unit. This would be a 100 per cent increase based on the lower limit and a 50 per cent increase based on the upper limit of untreated glands. The results obtained are consistent, and the method has been used successfully with rats derived from a Sprague-Dawley strain.

This method can be used to determine LH in unfractionated extracts if the injections are properly carried out.[43] If the injections are made subcutaneously, the true value of LH is not obtained in an unfractionated extract because FSH increases the responses to LH. But if an unfractionated extract is injected intraperitoneally, this augmentation does not take place when amounts ordinarily used for standardization are given, and the response is due primarily to the LH present. Pure LH, however, gives approximately the same result whether injected subcutaneously or intraperitoneally.

This method has been used in our laboratory with consistently reliable results, but the reports of other investigators indicate that the response to LH may not be alike in different strains of rats. Evans and coworkers find it difficult to produce any significant hypertrophy of the seminal vesicles with LH or with unfractionated extracts.[12] A preparation of LH furnished us by Doctor Evans—which in his animals (Long-Evans strain) produced very little seminal vesicle hypertrophy—quite readily produces as much as 600 per cent increase in seminal vesicle weight in our rats (Sprague-Dawley strain). Similarly, an unfractionated extract containing both FSH and LH produced maximal hypertrophy of the seminal vesicles in Sprague-Dawley rats but only about 30 per cent increase in Long-Evans rats. This is all the more surprising because Long-Evans rats do respond to implants of pituitary tissue.

Similarly, van Dyke and coworkers[44,45] found that in hypophysectomized rats of the Long-Evans strain the seminal vesicles do not respond markedly to LH injections but that the anterior lobe of the prostate serves as a better index of LH activity. Consequently they have used it as the end point. Their unit is described as an amount of material which produces a significant increase in the fresh weight of the anterior lobe of the prostate of rats hypophysectomized at 21 days of age. Injections are started 2 days after hypophysectomy and continued for 4 days, injecting once daily. Necropsy is performed 24 hours after the last injection.[18]

Evans and coworkers use hypophysectomized female rats to determine the activity of preparations of the luteinizing hormone.[12] The rats are

[43] **Fevold, H. L.** Jour. Biol. Chem. **28**: 83. 1939.
[44] **Greep, R. O.** Proc. Am. Physiol. Soc. in press.
[45] **Greep, R. O., van Dyke, H. B. & Chow, B. F.** Jour. Biol. Chem. **133**: 289. 1940.

hypophysectomized when they are 26 to 28 days old, and the injections are started 6 to 8 days after the operation. At this time the interstitial cells of the ovaries have assumed a deficiency condition. The rats are then injected intraperitoneally, once daily for 3 days with the preparation to be tested. At the end of the injection period the ovaries are removed, sectioned, and examined microscopically. The amount of the preparation which is sufficient to bring the interstitial cells back to normal is defined as the unit.

An assay method which is said to be a direct measure of LH, rather than indirect as those enumerated above, has been reported by Witschi.[34] The animal used is the African weaver finch. The seasonal breeding plumage of the males of these birds is determined directly by the gonadotropic hormones of the pituitary and more particularly by the luteinizing hormone. The female and the male plumage during the nonbreeding season is white. When LH is injected in the breast muscles, a black spot or bar appears in the feathers 2 days after injection; and the minimum amount to produce the black bar is taken as a unit. This effect is believed to be very specific for the LH, and the presence of FSH has very little, if any, effect on the results.

The unfortunate situation exists at the present time that no two laboratories have used the same method for LH assay, and there are no comparisons of the results obtained by the various methods. Correlation of the quantitative data dealing with the luteinizing hormone is urgently needed. Comparison is made still more difficult by the fact that not only are the methods different but strains of rats which seem to be of different physiological reactivity are used. Much confusion and misunderstanding would be avoided, and research on the luteinizing hormone would be greatly facilitated if some agreement could be reached on a method for standardization. It would be most practical if the assay method devised could be used on intact animals, thus obviating operative procedures such as hypophysectomy.

EXTRACTION AND PURIFICATION OF THE LUTEINIZING HORMONE

The pituitary tissue which has been used for chemical investigations of the gonadotropic hormones has been obtained almost exclusively from sheep and swine. Cattle pituitaries are available but contain very little gonadotropic material. The pituitaries are removed at slaughter houses and are preserved in one of two ways. They are either frozen and stored, or are desiccated with acetone, ground and stored as powders.

Either of these procedures has been thought to retain the hormones in active form.

A report has recently been made of an investigation dealing with various methods of preserving pituitary tissue.[46] The pituitaries were desiccated with acetone, with alcohol, air dried, and also stored in the fresh frozen state. The subsequent biological assays showed a marked loss of activity when desiccation was effected with acetone or alcohol, but only a slight loss when the tissue was dried in air. Storage of sheep pituitary tissue in the frozen state, at -10° C., resulted in no loss of gonadotropic activity after 80 days. From the results it appeared that autolysis of the tissue did not proceed very rapidly since no loss occurred on air drying. But the safest method for preservation and storage seems to be in the frozen condition.

Purification of the luteinizing hormone is effected by methods which are widely used in protein chemistry, namely: precipitation with salt, isoelectric precipitation, and by fractionation with aqueous acetone or alcohol. In the last two or three years a high degree of purification has been obtained in at least three laboratories. The preparations from two of these laboratories are reported to be pure proteins as determined by sedimentation in the ultracentrifuge, electrophoretic analysis and by solubility studies. The product obtained by the third group of workers appears not to be pure, as judged by these criteria, but seems to exceed those of the others in activity.

Van Dyke and coworkers used fresh hog pituitaries as their original material.[18,29,4] The method by which they obtain a pure protein preparation of the luteinizing hormone is as follows:

1. 1 kg. fresh hog pituitaries is extracted with 5 liters of cold 2 per cent NaCl.
2. pH is adjusted to 4.2–4.6 with N/70 HCl. Centrifuged.
3. Precipitate is washed twice with 2 per cent NaCl. 1.7 liters per washing.
4. Supernatants are combined and saturated with $(NH_4)_2SO_4$.
5. Precipitate is removed by filtration.
6. Precipitate is dialyzed in cold against distilled water until free of salt. Precipitate is removed and discarded.
7. pH is adjusted to 5.1. Precipitate is discarded.
8. Supernatants are brought to 50 per cent saturation with $(NH_4)_2SO_4$ at pH 4.2. Inactive precipitates are washed and discarded.
9. Solution is brought to 0.9 saturation with $(NH_4)_2SO_4$. Precipitate is collected by filtration and again dialyzed salt free.

[46] Kupperman, H. S., Elder, W. H. & Meyer, R. Endocrinology 29: 23. 1941.

10. One volume of 1M acetate buffer, pH 4.41 is added and two volumes of 41 per cent Na_2SO_4.
11. The precipitate is dissolved to a concentrated solution and the active material precipitated at ⅓ saturation $(NH_4)_2SO_4$, pH 7.3–7.4.
12. Precipitate is taken up in water and the active material precipitated by the addition of an equal volume of saturated ammonium sulfate, pH 7.3–7.4.
13. These last two steps are repeated 7 times and the final precipitate in a small volume of water is dialyzed salt free.

This material was found to contain 150 rat units per mg., 6.7 micrograms of the material being the minimal effective dose for the stimulation of the ventral prostate in hypophysectomized male rats.

Electrophoretic analysis of the material showed only one component to be present with a mobility of -3.85×10^{-5} at pH 4.58, -2.01×10^{-5} at pH 6.21 and 0.66×10^{-5} at pH 7.86. These measurements were made in acetate, cacodylate and diethyl barbiturate buffers of 0.05 ionic strength, and at temperatures near 0° C., the mobilities being corrected to 0°.

The average sedimentation constant in a 1 per cent NaCl solution was $s_{20} = 5.39$ S.* The diffusion constant, determined in a cacodylate medium, was found to be $D_{20} = 5.9 \times 10^{-7}$. From these values the molecular weight was estimated to be approximately 90,000.

Solubility studies likewise indicated that this material was a pure protein because the solubility was independent of the amount of solid present in excess of saturation.

Evans and coworkers[47] used acetone-desiccated sheep pituitary tissue as their original material, and proceeded with the extraction and purification of the active principle in the following manner:

1. Three hundred grams of acetone-dried whole sheep pituitary powder are extracted twice with 40 per cent ethyl alcohol (4 liters followed by 2 liters).
2. The active material is precipitated at alcohol concentration of 80–85 per cent, pH 5.5 with acetic acid.
3. The precipitate is dried with absolute alcohol and ether. Yield 12–15 grams.
4. Fifty grams of the powder are extracted with three liters of water. pH 4.5
5. The active material is precipitated with acetone. The precipitate

* All sedimentation constants are expressed in Svedberg units, denoted S, and equal to 10^{-13} sec.

47 **Li, C. H., Simpson, M. E. & Evans, H. M.** Endocrinology **27**: 803. 1940.

is extracted with one liter of 1 per cent saline solution and the insoluble material discarded.

6. The solution is brought to 0.5 saturation with $(NH_4)_2SO_4$ by addition of 1 volume of saturated $(NH_4)_2SO_4$ solution. The precipitate is redissolved and reprecipitated twice. The precipitate contains LH and the supernatant FSH.
7. Precipitate is made up to 800 cc. with water. 100 cc. of saturated $(NH_4)_2SO_4$ are then added. The precipitate is discarded.
8. The supernatant is brought to 0.4 saturation.
9. Steps 7 and 8 are repeated twice. Final precipitation is made from 500 cc.
10. The precipitate from 9 is dissolved in 300 cc. of solution and brought to 0.37 salt saturation by addition of 175 cc. saturated ammonium sulfate. Precipitate removed.
11. Salt concentration increased to 0.4 saturation. Precipitate contains active material.
12. Steps 10 and 11 repeated twice from smaller volume.
13. Final product was dissolved in water to a 1 per cent solution and 10 per cent trichloroacetic acid added to 2.5 per cent.
14. Precipitate is dissolved in small volume of alkaline solution and reprecipitated as in 13.
15. Final precipitate is dissolved in alkali and dialyzed against distilled water.
16. Precipitate was obtained in dry form by evaporation of a frozen block.

This material contains 100 to 200 rat units per milligram, the unit being based on the repair of the interstitial tissue of the ovaries of hypophysectomized rats. The yield of active material is but from 4 to 8 per cent if based on the activity of the starting material. It is free of all other pituitary hormones such as lactogenic, adrenotropic, growth, thyrotropic and the follicle-stimulating factors.

When this material was investigated electrophoretically it was found that after 90 minutes only one boundary was present. The mobility was 6.36×10^{-5} in phosphate buffer, pH 7.53, ionic strength 0.05 at 1.5° C.

In another publication[18] in which an alternate method of preparation is given, the sedimentation constant of the pure protein is given as 3.6 S in a 1 per cent NaCl solution. The isoelectric point is given as pH 4.6 and the molecular weight as 40,000. Solubility studies are also reported as an indication of the purity of the protein preparation.

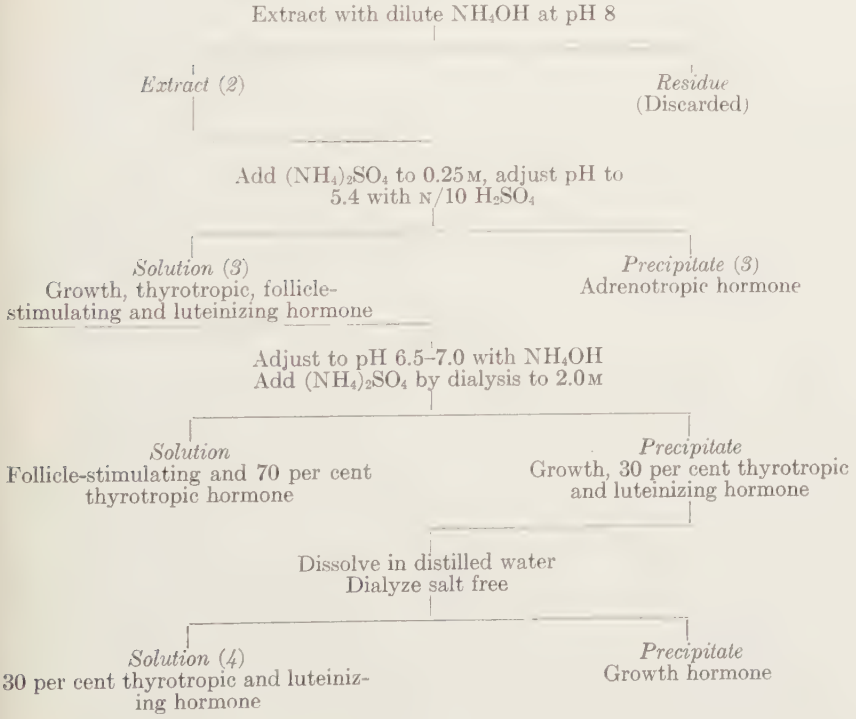
[18] **Li, C. H. & Evans, H. M.** Jour. Am. Chem. Soc. in press.

In the Harvard laboratories we have also attempted to carry out the fractionation and purification of the proteins of the anterior pituitary glands. This joint research was undertaken because of the wide experience with the hormones of this gland by certain of our colleagues and because of the conviction of others that there was "no theoretical obstacle to the isolation of all the protein constituents of any given tissue, or to their characterization as chemical substances, and to the study of their interactions as biological components."[55] "Only reagents and conditions which are known to have little, if any, deleterious effect on most proteins have been employed. The pH has been retained between 5.4 and 8.0. Only two ions, ammonium and sulfate, in cold aqueous solution, have been added throughout the process"[49] in its early stages. In further preparations phosphate buffers have been employed to control the pH, which was never less than 5.2.

[49] **Fevold, H. L., Lee, M., Hisaw, F. L. & Cohn, E. J.** Endocrinology **26**: 999. 1940.

Diagram 1

Fresh Frozen Sheep Pituitary Tissue (1)



Recognizing that generally, in studies of this kind, "the limiting factor was never the chemical study of the active principle, but the unsatisfactory nature of the methods for bio-assay," the direction and responsibility for all bio-assay in this problem was undertaken by the Harvard Biological Laboratories and for chemical fractionation and characterization by the Department of Physical Chemistry at the Harvard Medical School.

The first step in this investigation was the "Separation of Five Anterior Pituitary Hormones into Different Fractions by Isoelectric and Ammonium Sulfate Precipitation," reported in our previously published paper.[49] Diagram 1 presents schematically the extraction, concentration and gross separation of the luteinizing hormone from the adrenotropic, growth, thyrotropic and follicle-stimulating hormone.

Traces of protein in (4) precipitable at pH 5.4 may conveniently be removed at this point, the reaction being immediately readjusted to pH 7. Thereafter, besides the luteinizing hormone, the main remaining hormone is roughly 30 per cent of the thyrotropic, which is in our crude solution (4). The thyrotropic hormone is more soluble in ammonium sulfate than is the luteinizing hormone. It can thus be removed by repetition of the precipitation with ammonium sulfate at the appropriate salt concentration yielding our final fraction (4).

Table 2 gives the solids and yields of various hormones in the first three steps of the procedure. Preparations III, IV, V and VII were prepared in the Harvard laboratories[50]. We are indebted to G. H. A. Clowes and J. C. Leighty for carrying out the extraction and initial fractionation of preparations XI, XII, XIII and XIV at the Eli Lilly and Company Laboratories.

Although the solids in solution (3) as prepared by J. C. Leighty were somewhat higher than in our smaller preparations, so, in many cases, were the activities, and it was a great satisfaction to discover that the process we had employed could readily be carried out by independent workers in another laboratory. The salting out of various hormones by ammonium sulfate has been carried out with great care by dialyzing the electrolyte through rotating collodion membranes at constant temperature and pH. By avoiding local excess of reagents by this procedure, denaturation of the various proteins is much diminished on the one hand; on the other, far cleaner separations between the proteins present in each

[50] Fevold, H. L., Oncley, J. L., Armstrong, S. H., Lee, M., Hisaw, F. L. & Cohn, E. J. Unpublished studies.

TABLE 2
EXTRACTION OF ANTERIOR PITUITARY HORMONES*

	Preparation	Solids[a]	Adrenotropic hormone	Growth hormone	Luteinizing hormone	Thyrotropic hormone	Follicle-stimulating hormone[b]
		gm/kg	*RU/kg*	*RU/kg*	*RU/kg*	*CU/kg*	*RU/kg*
pH 8.0 Extract (2)	III	59.8		50,000	8,000		(2,500)
	IV	58.0		52,000	12,000	2,000	(3,000)
	V	82.5	5,000	52,000	18,000	3,000	(2,000)
	VII	71.2	5,000	50,000	20,000	4,000	(3,000)
pH 5.4 Precipitate (3) in 0.25 M $(NH_4)_2SO_4$	III	40.4			none[c]	none[c]	none[c]
	IV	35.4	5,000	none[c]	none	none	none
	V	59.4	5,000	none	none	none	none
	VII	48.1	5,000	none	none	none	none
pH 5.4 Solution (3) in 0.25 M $(NH_4)_2SO_4$	III	19.4			8,000		(2,500)
	IV	22.6	none[c]	76,000	14,000	2,000	(3,000)
	V	22.9	none	112,000	16,000	3,000	(2,000)
	VII	23.8	none		20,000	4,000	(3,000)
	XI	32.9	none	74,000	26,000	4,000	
	XII	36.0	none	43,000	13,000	2,500	(6,000)
	XIII	28.0[d]	none	36,000[d]	8,000[d]	4,500	(6,000)
	XIV		none		48,000	4,000	(5,000)

[a] All solids were determined by dry weight of aliquots after dialysis. Assay units are per kg. of fresh gland.
[b] Values given are only approximations, calculated from the number of units of follicle-stimulating hormone recovered after separation from luteinizing hormone. No method is available for the accurate determination of follicle-stimulating hormone in the presence of luteinizing hormone.
[c] The term "none" is applied when the response was unsatisfactory to maximum amounts of material reasonably tolerated by the test animal.
[d] One fraction of this preparation was lost in shipment.
* Bio-assays of the growth hormone were carried out by Milton Lee, who had collaborated throughout in this phase of our anterior pituitary hormone investigations. Bio-assay of the thyrotropic hormone was carried out by A. A. Abramowitz, and for the luteinizing and follicle-stimulating hormones by H. L. Fevold, both in collaboration with F. L. Hisaw in the Biological Laboratories.

system are effected at each ionic strength.[51-54] The results of this process as carried out in our earlier preparations have already been published.[55] Comparable studies with preparations XI, XII and XIII are reported in TABLE 3. Although there is considerable deviation in the results from preparation to preparation, this comparison of four different preparations reveals that the previously reported separations can readily be carried out on a far larger scale than we have heretofore reported.

By far the highest activity of the luteinizing hormone was found in the fractions precipitated by ammonium sulfate at 1.6 and 1.8M respectively. Indeed, in preparations XI, XII and XIII, less than 1,000 RU/kg. were found in the fractions precipitated beyond 2M ammonium sulfate. Moreover, the amounts of solids in these most active fractions were smaller than those from the soluble proteins precipitated by ammonium sulfate at either the lower ammonium sulfate concentration of 1.2 or at concentrations beyond 2.4. We therefore carried out the further purification of the luteinizing hormone by again precipitating the uncombined water-soluble proteins precipitated respectively at 1.2, 1.4, 1.6 and 1.8M ammonium sulfate. As an example of procedure we shall give in detail the purification of preparation XI derived from 6 kg. of frozen tissue. The total solids in this preparation at this point were 21.3 grams and the activity in RU/kg. was estimated to be 6.5, or within the levels that we have previously reported[56] for this crude luteinizing hormone fraction of from 5–10 RU/mg. Several preparations have been more potent at this stage.

The above solution was again fractionated with ammonium sulfate at pH 6.8–7.0, precipitates removed respectively at ammonium sulfate concentration of 1.3, 1.95 and 2.6, the ammonium sulfate being added through a cellophane membrane as before. At 1.3M, 1.4 grams of protein precipitated. At 1.95, 11.5 grams precipitated and at 2.6, 6.4 grams. The first fraction contained less of the luteinizing hormone than we could test for; the last only traces. The fraction precipitating from 1.3–1.95 contained just over half of the protein, thus containing by far the greater part of the activity.

The middle fraction was again dissolved and precipitated with am-

[51] **Cohn, E. J., McMeekin, T. L., Oncley, J. L., Newell, J. M. & Hughes, W. L.** Jour. Am. Chem. Soc. **62**: 3386. 1940.
[52] **Evans, H. M., Simpson, M. E. & Pencharz, R. I.** Cold Spring Harbor Symposia on Quant. Biol. **5**: 229. 1937.
[53] **Evans, H. M.** Addresses at the Dedication of the Research Building of Abbott Laboratories, p. 20.
[54] **Greep, R. O., van Dyke, H. B. & Chow, G. F.** Am. Jour. Physiol. **133**: 303. 1941.
[55] **Cohn, E. J.** Bull. N. Y. Acad. Med. **15**: 639. 1939.
[56] **Pillemer, L., Ecker, E. E., Oncley, J. L. & Cohn, E. J.** Jour. Exp. Med. **74**: 297. 1941.
[57] **McMeekin, T. L.** Jour. Am. Chem. Soc. **61**: 2884. 1939.
[58] **McMeekin, T. L.** Jour. Am. Chem. Soc. **62**: 3393. 1940.
[59] **Cohn, E. J., Luetscher, J. A., Jr., Oncley, J. L., Armstrong, S. H., Jr. & Davis, B. D.** Jour. Am. Chem. Soc. **62**: 3396. 1940.

TABLE 3

FRACTIONATION OF pH 5.4, 0.25 M $(NH_4)_2SO_4$-SOLUBLE PITUITARY EXTRACTIVES WITH $(NH_4)_2SO_4$ AT pH 6.5–7.0

Concentration of $(NH_4)_2SO_4$	Globulins precipitated	Growth hormone	Soluble proteins	Luteinizing hormone	Thyrotropin	Follicle-stimulating hormone
mols/L	*gm/kg*[a]	*RU/kg*	*gm/kg*[a]	*RU/kg*	*CU/kg*	*RU/kg*
		(See Table 2 and Ref. 49)				
1.4	6.13	25,000	0.51	2,000	none	none
1.6	1.66	64,000	0.31	4,000	none	none
1.8	1.21	trace	0.63	4,000	300	none
2.0	0.64	none	0.61	4,000	350	none
2.2	0.34	none	0.49	1,000	1,500	none
2.4	0.32	none	0.66	500	trace	trace
2.6	0.28	none	0.81	trace	none	1,000
2.8	0.40	none	0.75	none	none	2,000
3.8	1.88	none	2.33	none	none	none
			Preparation XI			
1.2	11.35	11,000	1.05	3,000	200	none
1.4	4.80	25,000	0.60	10,000	100	none
1.6	2.40	26,000	1.00	4,000	300	none
1.8	1.26	2,000	0.90	6,000	550	none
2.4	2.05	5,000	3.11	—	2,500	traces
3.0	2.21	none	2.14	—	100	1,000
			Preparation XII			
1.2	12.05	—	0.75	1,000	none	—
1.4	4.50	76,000	0.35	4,000	none	—
1.6	3.84	6,000	0.46	4,000	500	—
1.8	3.34	3,800	0.62	6,000	600	—
2.4	2.35	2,800	—	—	1,300	2,000
3.0	2.31	—	—	—	—	4,000
			Preparation XIII			
1.2	9.28	4,400	0.80	800	none	—
1.4	*	*	*	—	—	—
1.6	2.96	6,300	0.94	2,000	800	—
1.8	2.96	2,400	0.74	4,000	800	traces
2.4	2.24	—	3.80	1,000	2,800	2,000
3.0	2.30	—	—	—	none	4,000

[a] Dry weight of aliquots after dialysis. Assay units are given per kilogram of fresh gland.
* Lost in shipment.

monium sulfate from a still smaller volume. Since the total amount of protein in this system was smaller, the volume was also maintained smaller and fractions removed respectively at 1.4, 1.7 and 2.6 M. The first and the last contained only traces of the luteinizing hormone, which was thus again largely concentrated in the middle fraction which contained 3.9 grams. Since the total solids were reduced from 21.3 to 3.9 grams, an increase in activity from 6.5 to 35.5 RU/mg. would result were the yields of luteinizing hormone quantitative. Actually, the losses in

carrying out this process have always been small, yielding fractions with activities varying from 30 to 40 RU mg. as previously reported.[19]

Further concentration of the luteinizing hormone has been accomplished by dissolving the material with activities of 30–40 RU mg. in water, dialyzing salt free and carefully adjusting to pH 5.0–5.2 with dilute H_2SO_4. A precipitate with comparatively small activity (about 10 per cent of the total) was removed. The supernatant solution was precipitated with $(NH_4)_2SO_4$ at pH 7 in order to concentrate the protein, redissolved in about 17 cc. of water per kilogram of fresh tissue, and again dialyzed salt free and adjusted to pH 5.0–5.2, when a further precipitate was removed. This entire process was then repeated a third time. Approximately one quarter of the original protein remained in solution at pH 5.2 after this third precipitation from small volume. Were the yield of hormone quantitative, an activity of from 120–160 RU mg. would be achieved. Bio-assay revealed losses of from 10 to 20 per cent; the activity achieved should thus have been of the order of 100–140 RU mg. Actually, bio-assays have revealed even greater activity at this stage, and the solids from 6 kilograms of fresh frozen sheep pituitary glands have been reduced to less than one gram.

This material was studied electrophoretically in a phosphate buffer of pH 7.7 and ionic strength 0.2. The schlieren diagram obtained reveals two and perhaps three components with mobilities of approximately 2.0, 4.0 and 5.4×10^{-5} cm²/volt sec.

This concentrated solution has been the starting material in our further efforts to purify the luteinizing hormone. It was next dialyzed against phosphate buffer of pH 5.6 and 0.25 ionic strength until equilibrium was reached, and then dialyzed against distilled water and finally conductivity water. The pH was then 5.9–6.0. Under these conditions a euglobulin separated from the concentrated solution. The precipitated globulin was removed, and the supernatant solution concentrated by negative pressure dialysis to a small volume (15 cc. per kg. of frozen tissue). This was further chilled to 0° C. and the precipitated globulin combined with the main globulin precipitate.

The precipitated globulin, in a volume of 1 cc. kg. of frozen tissue, was extracted with a small quantity of 1.18M ammonium sulfate, at pH 6 and 0° C. This solution was separated from the undissolved precipitate by centrifugation at 0° C. and then brought to 25° C., when a precipitate containing most of the luteinizing hormone separated. The supernatant was next cooled to 0° C. and employed in a second extract of the original precipitate again at 0° C. This process was repeated as often as any material was precipitated at 25° C. under these conditions. The pre-

cipitates obtained by the increase in temperature were collected and contained luteinizing hormone.

Active material was thus obtained as, or associated with, a globulin with a negative temperature coefficient between 0° and 25° C. at pH 6 in 1.16M ammonium sulfate. The amount of material obtained at this point was roughly of the order of 10 mg. kg. of fresh pituitary tissue. The fractions with activity above 150 RU/mg. have not been prepared sufficiently often for us to report values for the activity of this globulin at this time. Although great significance cannot be attached to the physical constants of this fraction since it has not been prepared often enough, in the interests of other workers in the field it may be helpful to report the ultracentrifugal and electrophoretic measurements that have been made.

Examination in the ultracentrifuge carried out by J. L. Oncley over a considerable range of protein concentration showed that the protein in this globulin fraction was not homogeneous with respect to size and shape. Components of different sedimentation constant were present in a 1.3 per cent solution in 0.2M KCl. A slower moving component comprised approximately 60 per cent of the total and had a sedimentation constant of about 3.3 S, and the remainder of the material had a sedimentation constant of about 6.6 S. A preliminary study in a divided ultracentrifuge cell indicated that the fraction of lower sedimentation constant was active. Whether the fraction of higher sedimentation constant was active has not been established.

Electrophoretic analysis of this globulin fraction at a protein concentration of 0.4 per cent in phosphate buffer of pH 7.7 and ionic strength 0.2 also revealed at least two components. Of these, the slower moving, estimated to be about 75 per cent of the total, had an electrophoretic mobility of about 1.8×10^{-5}, while the faster moving component, comprising approximately 25 per cent of the total, had a mobility of about 5.3×10^{-5}. Electrophoretic separation of the two components, carried out by S. H. Armstrong, Jr., suggested that the fast moving material was relatively inactive while the slow moving fraction appeared the more active.

These observations thus suggested that the highest activity we have noted was in the component of smaller sedimentation constant and lower electrophoretic mobility. It is always possible, however, that none of the protein components revealed by the schlieren diagrams represent the active hormone.

This work was interrupted at this point by my acceptance of a govern-

ment post and repetition of the experiments at Harvard has been postponed because of preoccupation with protein problems related to defense.

COMPARISON OF LUTEINIZING HORMONE PREPARATION

A comparison of the physical constants of the luteinizing hormone preparations from the three laboratories is presented in TABLE 5. It is apparent that if we assume that the proteins isolated and studied by the Squibb and Rockefeller Institute group, and by the University of California group, are pure luteinizing hormone proteins, we must come to the conclusion that sheep and swine luteinizing hormones are two different proteins. Chemical analysis of the two isolated proteins from sheep and swine pituitaries also brings out the fact that they are different proteins. The sheep protein isolated by the University of California group contains 4.5 per cent mannose, 5.8 per cent glucosamine, 1 per cent tryptophane, 4.5 per cent tyrosine, and 5.4 per cent cystine. The protein isolated by the Squibb and Rockefeller Institute group from swine pituitary contains only 2 per cent carbohydrate, but 3.8 per cent tryptophane. The other constituents mentioned above have not been determined for swine preparations.

TABLE 5
COMPARISON OF LUTEINIZING HORMONE PREPARATIONS

	Isoelectric point pH	Electrophoretic mobility 0° C., $\times 10^5$	Sedimentation constant. Svedberg Units, S	Molecular weight
Squibb and Rockefeller Institute (fresh swine pituitary)	7.4	0.6[a]	5.4[d]	90,000[f]
University of California (acetone-dried sheep pituitary)	4.6	6.4[b]	3.6[d]	40,000[g]
Harvard University (fresh sheep pituitary)	—	1.8–2.0[c]	3.3; 6.6[e]	

[a] In 0.005 ionic strength, diethylbarbiturate buffer, pH 7.86.
[b] In 0.05 ionic strength, phosphate buffer, pH 7.53.
[c] In 0.02 ionic strength, phosphate buffer, pH 7.77.
[d] In 1 per cent (0.17M) NaCl.
[e] In 0.20M KCl.
[f] From sedimentation velocity and diffusion measurement.
[g] From osmotic pressure measurements.

If we consider the work on sheep pituitary tissue, the agreement is also not too satisfactory. The sedimentation constant of our slow sedimenting component is close to that reported by the University of California workers. The electrophoretic mobilities reported by the two

laboratories do not agree, and the difference would appear greater than that expected from the difference in ionic strength and protein concentration used in the separate investigations. The higher mobility observed by the University of California workers is of the same magnitude as the faster moving electrophoretic component of relatively low activity observed in our work, however.

The activity data are even more difficult to reconcile, and would appear to be greater than can be explained by the different methods of bio-assay employed in different laboratories.

NOMENCLATURE OF THE LUTEINIZING HORMONE

In conclusion, a few words should be said regarding the nomenclature of the luteinizing hormone. The pituitary gonadotropic hormones were originally given names which defined their physiological actions in the female, namely follicle-stimulating and luteinizing hormones (FSH and LH).[5–7] It was subsequently shown that these two factors stimulated spermatogenesis and interstitial-cell hypertrophy respectively in the male.[10 11]

Evans and coworkers reported that the factor which stimulates the interstitial cells was not the factor which produces luteinization. They named this new substance the interstitial-cell-stimulating hormone (ICSH) and reported a complete separation of ICSH and LH.[9,52] This claim was later withdrawn[53] but the term ICSH has been retained by them for the substance which produces both luteinization and interstitial-cell stimulation, in other words for the luteinizing hormone. The terms LH and ICSH are now synonymous and refer to the same substance.

The Squibb group has recently preferred to devise Greek names for the two substances and have called them "thylakentrin" and "metakentrin"; thylakentrin referring to the FSH and metakentrin to the LH.[54]

The luteinizing hormone is therefore identified with three names in the literature: LH, which was the name originally given to the hormone, ICSH, and metakentrin.

THE LACTOGENIC HORMONE AND MAMMOGEN*

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Section I: The Lactogenic Hormone

INTRODUCTION

The first experimental evidence for the presence of a hormone or hormones in the anterior pituitary body which are essential for the initiation and maintenance of lactation was presented by Stricker and Grüter[1] in 1928. The existence of a specific, anterior lobe, lactogenic hormone is generally accepted at the present time. Various names have been proposed for this hormone: prolactin (Riddle), galactin (Turner), and mammotropin (Lyons). It appears that prolactin has been most widely used. It is not intended in the present discussion to consider whether or not prolactin is *the* lactogenic hormone; the term prolactin will be employed to designate *a* lactogenic hormone of the anterior pituitary. The ability of this substance to initiate, in a variety of species, lactation in glands presumably possessing a certain amount of alveolar development has been established by numerous investigators.[2–4]

The fact that prolactin was the first anterior pituitary hormone to be obtained in a highly purified state and as an apparently homogeneous preparation resulted in a considerable accumulation of physical-chemical and chemical data for this hormone. In order to permit adequate discussion of this material, the physiological aspects of prolactin will not be detailed. It may be added that the physiologists have far outstripped the chemists in the rate of production of new facts regarding the anterior pituitary hormones. Consequently, there have been exhaustive treatments of the physiology of prolactin,[2, 5] and repetition of this material at the present time would contribute little to this symposium. The methods of assay and preparation of prolactin will be only mentioned, and detailed descriptions of experimental procedures by which other data were obtained will be left for presentation elsewhere.

* Some of the data presented in this publication were obtained in investigations aided by grants to the author from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association, and from the Fluid Research Fund of the Yale University School of Medicine.

1 **Stricker, P., & Grüter, F.** Comp. rend. Soc. Biol. **99**: 1978. 1928.

2 **Nelson, W. O.** Physiol. Rev. **16**: 488. 1936.

3 **Turner, C. W.** In "Sex and Internal Secretions," 2nd edit., E. Allen, Ed. Williams and Wilkins Co. Baltimore. 1939. p. 740.

4 **Folley, S. J.** Biol. Rev. **15**: 421. 1940.

5 **Riddle, O., & Bates, R. W.** In "Sex and Internal Secretions," 2nd. edit., E. Allen, Ed. Williams and Wilkins Co. Baltimore. 1939. p. 1088.

It is desired to acknowledge gratefully the stimulating collaboration and support given this work by Professor C. N. H. Long. Dr. R. W. Bonsnes contributed to certain of the data obtained for purified prolactin, which are to be discussed. Dr. H. R. Catchpole conducted some of the early bio-assays and Miss Hilda B. Ritter subsequently collaborated in the many bio-assays which have been done in the course of this work. Studies on the concentration and isolation from natural sources of substances which may occur in relatively small amounts are greatly facilitated by the cooperative efforts of a number of individuals. This is particularly emphasized when progress must be evaluated by biological assays which are often laborious.

METHODS OF ASSAY OF PROLACTIN

The methods generally employed for the assay of prolactin may be classified into two groups: (1) crop-sac or crop-gland methods, and (2) lactation or mammary gland methods. These methods have been discussed in detail by Bates,[6] Riddle and Bates,[5] and Lyons.[7a] The two laboratories represented by these workers, and that of the National Institute for Research in Dairying at Reading, England, as exemplified in the investigations of Folley and coworkers,[8] have examined in great detail factors influencing the assay of prolactin. These descriptions, together with the availability of an International Standard of an accepted unitage makes possible the highly desirable comparison of products obtained by various investigators.

Prolactin activities in the work to be described have been determined by two methods: (a) the 2-day "local" assay, and (b) the 4-day systemic test.[7] It has been observed that the systemic method has given more reproducible and consistent results, and this technique has therefore been generally employed. Six-weeks-old pigeons of a single strain (White Carneau) and from a single source (Palmetto Pigeon Plant, Sumter, South Carolina) have been used. Comparisons have been made with the International Standard, kindly supplied by Dr. A. S. Parkes, with an accepted activity of 10 International Units per mg. It may be mentioned that, in agreement with the recent publication of Lyons,[7b] it has been found that the activity claimed for the International Standard is approximately three times too high. Our preparations were initially evaluated in terms of absolute activity and the impression therefore arose that certain amorphous prolactin preparations were approximately

[6] **Bates, R. W.** Cold Spring Harbor Symposia on Quantitative Biology **5**: 91. 1937.
[7a] **Lyons, W. R.** Cold Spring Harbor Symposia on Quantitative Biology **5**: 198. 1937.
[7b] **Lyons, W. R.** Endocrinology **28**: 161. 1941.
[8] **Folley, S. J., Dyer, F. J., & Coward, K. H.** Jour. Endocrinology **2**: 179. 1940.

two to three times as active as the products we obtained. It is of course necessary and desirable, for purposes of comparison with other investigators, to accept the value of 10 International Units per mg. for the International Standard in evaluating the activity of unknown products. This has been done in obtaining bio-assay data presented here.

PREPARATION OF PROLACTIN

Extracts highly active in prolactin may be prepared from either fresh or acetone-desiccated pituitary glands by acid or alkaline extraction, with aqueous or aqueous-organic solvents. Bergman and Turner[9] compared 4 frequently employed methods of extraction of the lactogenic hormone and found the alkaline 60 to 70 per cent ethanol extraction procedure to be superior for both total yield and unitage per mg. of extracted solids. Though it is true that an alkaline extraction procedure is efficient in removing a very considerable proportion of the prolactin present in pituitary tissue, alkaline solvents also effect solution of relatively large amounts of extraneous tissue proteins. For this reason, the acid-acetone technique of Lyons[10] has been employed in the investigations in our laboratory. This excellent method is relatively simple and gives a product from which a highly purified preparation of prolactin may be obtained. The chief attribute of Lyons' method is the fact that the crude prolactin obtained is quite free from other pituitary proteins, the chief contaminant being one other pituitary hormone of considerable interest, namely, the adrenotropic factor.

One kg. of whole beef pituitary glands is extracted as described by Lyons.[7a,10] The acetone concentration of the acid-acetone extract obtained is increased to 92 per cent and the mixture allowed to stand overnight in the icebox. The precipitate is separated by centrifuging. Some purification is effected by extraction of the precipitate with 4 50 ml. portions of water, separating the extract each time from a water-insoluble residue by centrifuging, combining the clear extracts and adding acetone to a concentration of 92 per cent. After being chilled overnight the precipitate is separated by centrifugation, washed thoroughly with acetone and dried *in vacuo* over sulfuric acid at room temperature. Range of yields from 1 kg. of glands is 1.2 to 1.9 gm.

Purification of Prolactin

The product obtained as described contains a considerable quantity of protein which exhibits a minimal solubility at pH 6.4 to 6.8. This is

[9] **Bergman, A. J., & Turner, C. W.** Jour. Biol. Chem. **118**: 247. 1937.
[10] **Lyons, W. R.** Proc. Soc. Exp. Biol. Med. **35**: 645. 1936–37.

the so-called adrenotropic fraction.[10] A complete separation of the latter material from prolactin may be effected by solution of the crude product at approximately pH 8.0, with the aid of 0.1 N sodium hydroxide, adjusting to pH 6.6 by the addition of 0.1 N hydrochloric acid, and removing the precipitate which forms. Prolactin is obtained from the supernatant by lowering the pH to 5.4 by further addition of the dilute acid. The precipitate of prolactin is then redissolved in dilute alkaline solution as before and any precipitate which separates above pH 6.0 is removed. By repeating this procedure several times, and gradually reducing the volumes of the solutions employed, a very effective purification of prolactin results. Better separations of adrentropic protein and prolactin, and somewhat larger yields of the latter have been obtained by the use of ammonium sulfate together with adjustment of hydrogen ion concentration. Both the adrenotropic and prolactin fractions have a significant degree of solubility at the hydrogen ion concentrations at which they exhibit maximum flocculation. The presence of salt diminishes loss of prolactin in supernatants, and at the same time increases the completeness of separation of the adrenotropic fraction. A flow sheet indicating the preparative procedure employed is shown in CHART 1.

CRYSTALLIZATION OF PROLACTIN

Several years ago a preliminary communication from this laboratory[11] reported that a crystalline protein had been obtained from a highly purified prolactin product prepared by a procedure essentially that described by Lyons.[7, 10] The yield of crystalline material was small, due chiefly to denaturation of the protein which occurred during the crystallization procedure. This fact, together with subsequent observations that the original procedure described[11] has not been uniformly successful, has rather diminished emphasis on crystallization of the protein. This has been particularly true in view of the fact that, in the case of proteins, the crystalline state is not an adequate criterion of purity. Indeed, recrystallization of prolactin has frequently been accompanied by a decrease in biological potency and a diminished solubility of the protein. Similar observations have been made independently in the Schering Laboratories by Dr. Erwin Schwenk and Dr. Gerhard Fleischer, who have informed the writer that they have been successful in obtaining crystalline preparations of prolactin.

Two methods have been employed for obtaining crystalline products.

[11] White, A., Catchpole, H. R., & Long, C. N. H. Science 86: 82. 1937.

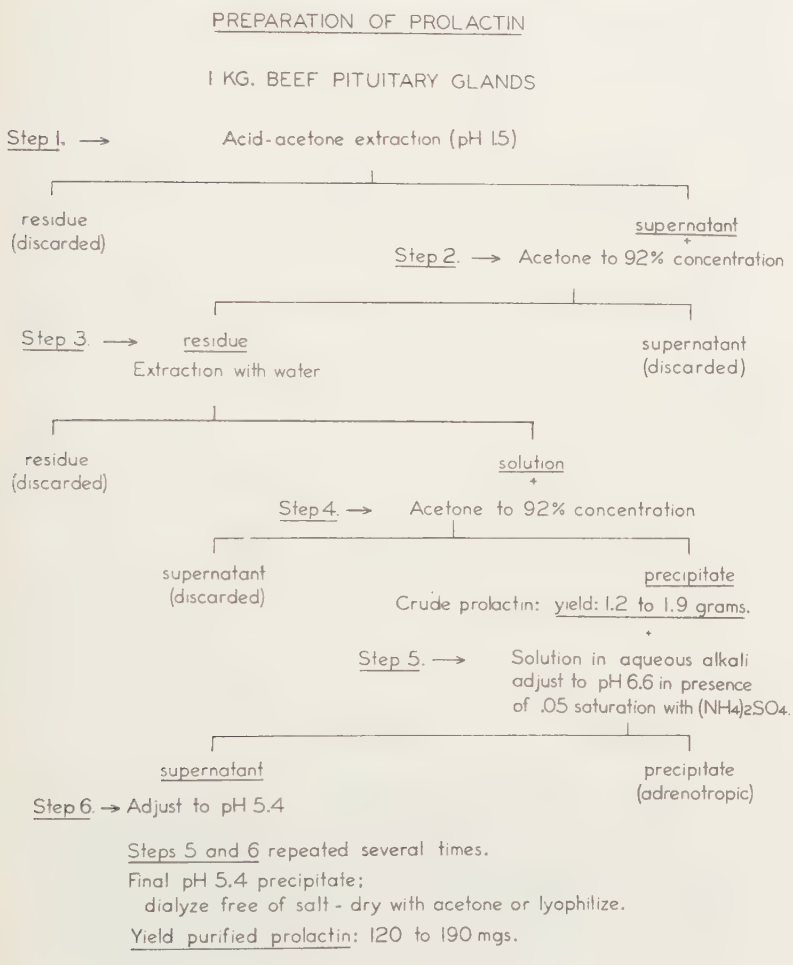

CHART 1. Preparation of Prolactin.

viz., (a) an acetic acid-pyridine procedure which is essentially that described[12] for the crystallization of insulin, and (b) precipitation from dilute acetone solutions. Some variation in concentrations or acetic acid and pyridine, as compared to those prescribed for the insulin crystallization, has been used because clearer supernatants, from which crystals deposit, are obtained. Dr. Schwenk states that the crystallization method employed in the Schering Laboratories is essentially the tech-

[12] **du Vigneaud, V., Jensen, H., & Wintersteiner, O.** Jour. Pharm. Exp. Ther. **32**: 367. 1927–28.
[13] **Abel, J. J., Geiling, E. M. K., Rouiller, C. A., Bell, F. K., & Wintersteiner, O.** Jour. Pharm. Exp. Ther. **31**: 65. 1927.

nique published by Abel and his colleagues,[13] with the modification of omitting brucine from the procedure.

A few remarks may be made regarding the crystallization procedures:

(1) Either technique yields crystalline material which is essentially the same in microscopic appearance. The latter is most frequently that of small hexagonal crystals. Clearer definition of crystalline form has been obtained after longer periods of standing at low temperatures, although this observation is not a consistent one. The preparations under the microscope frequently have the appearance of prismatic crystals, the edges of which are somewhat rounded. The crystals tend to dissolve slowly as the solution warms to room temperature. A photomicrograph of a preparation which had been permitted to deposit over a 2-month period at low temperature is shown in FIGURE 1.

(2) The yield of crystalline product is exceedingly small. In the case of the pyridine-acetic acid procedure the small yield may be attributed largely to the fact that purified prolactin appears rapidly to lose its solubility when subjected to repeated re-solutions in dilute acetic acid; this is necessary to get some of the protein into the pyridine-acetic acid mixture. The insoluble residue remaining after 10 reprecipitations of the protein, as is done in the crystallization method, amounts to 50 to 65 per cent of amorphous prolactin taken. Bio-assay of this insoluble residue indicates that a loss of 50 to 80 per cent of the physiological potency generally

FIGURE 1. Crystalline prolactin. × 900 diam.

accompanies loss in solubility of prolactin occurring in this step of the crystallization procedure. The limited solubility of the prolactin under the conditions employed has contributed to the low yield of crystalline protein.

(3) Recrystallization has not been satisfactory because of apparent denaturation of the protein, associated frequently with a decrease in biological potency. Recrystallizations which have been successful have been carried out by the pyridine-acetic acid procedure.

(4) The dilute acetone technique suffers also from the drawback of low yield of crystalline product. The procedure takes advantage of the fact that prolactin exhibits a considerable degree of solubility when precipitated from slightly alkaline solution by adjustment of the hydrogen ion concentration to the point of maximum flocculation. The protein remaining in solution may be precipitated from the supernatant fluid by the addition of acetone to a concentration of 80 per cent, followed by chilling of the solution.

The prolactin activities of the various products are presented in TABLE 1. For purposes of comparison, two prolactin preparations from other laboratories have been carefully assayed. Each of these was prepared from sheep pituitary glands; one was kindly furnished by Dr. Schwenk and the other was obtained through the generosity of Dr. W. R. Lyons of the University of California. It will be seen from the data in TABLE 1 that several preparations of purified prolactin, prepared in three different laboratories, have a biological activity which, within the limits of error of the assay method, may be considered identical.

TABLE 1

PROLACTIN ACTIVITY OF VARIOUS PREPARATIONS

Preparation	International Units per mg.
Crude prolactin	10–15
Purified prolactin	30–35
Crystalline prolactin (pyridine-acetic acid procedure)	30–35
Crystalline prolactin (acetone procedure)	30
Twice recrystallized prolactin (pyridine-acetic acid procedure)	30–35
Purified prolactin*	30–40
Purified prolactin**	30

* Obtained from Dr. Schwenk.
** Obtained from Dr. Lyons.

HOMOGENEITY STUDIES

The methods generally accepted as useful for examining the homogeneity of proteins are (a) the Tiselius electrophoresis technique, (b) solubility studies, and (c) ultracentrifugal analysis. Each of these methods has been employed in the study of prolactin. All preparations have been obtained from beef pituitary glands.

Electrophoresis in the Tiselius Apparatus

The electrophoretic behavior of crystalline prolactin has been previously reported from this laboratory.[14] Since the initial publication, the apparatus has been equipped with the schlieren scanning device of Longsworth.[15] FIGURE 2 shows scanning patterns obtained in a typical experiment with a crystalline prolactin preparation (35 I.U. mg.). The protein boundary migrated in a manner characteristic of a homogeneous protein. Similar homogeneity was observed in an electrophoresis experiment conducted at pH 3.90 (acetate buffer; ionic strength 0.05).

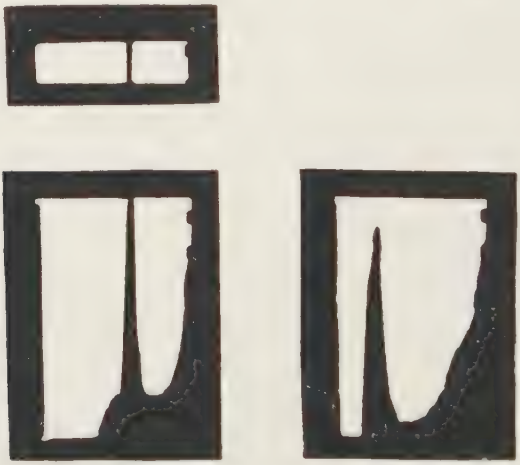

FIGURE 2. Electrophoretic patterns of the descending boundary in an experiment with crystalline prolactin. Protein concentration, 2 per cent; buffer, 0.017 M phosphate (pH 8.0); ionic strength, 0.1; temperature, 6° C. Photographs, from left to right, at zero time and after 4 hours.

The homogeneity and mobility of highly purified prolactin preparations from both beef and sheep pituitary glands have been studied in some detail by Li, Lyons, and Evans.[16] These investigators have demon-

[14] **Shipley, R. A., Stern, K. G., & White, A.** Jour. Exp. Med. **69**: 785. 1939.
[15] **Longsworth, L. G.** Jour. Am. Chem. Soc. **61**: 529. 1939.
[16a] **Li, C. H., Lyons, W. R., & Evans, H. M.** Jour. Gen. Physiol. **23**: 433. 1940.
[16b] Jour. Am. Chem. Soc. **62**: 2925. 1940.
[16c] Jour. Biol. Chem. **140**: 43. 1941.

strated that the lactogenic products prepared from these two different species are homogeneous and indistinguishable in their electrophoretic behavior.

Solubility Studies

Solubility studies supporting the conclusion that their highly purified, amorphous prolactin is homogeneous have been published by Li, Lyons, and Evans[17] who reported that although both sheep and beef prolactin preparations behaved as pure substances, they exhibited differences in solubility, thus indicating a species specificity. Crystalline prolactin with a physiological activity of 30 to 35 International Units per mg. has been studied in 3 series of solubility experiments, each with a different solvent: (a) 0.12M NaCl in 0.01N HCl, (b) 0.33M NaCl in 0.01N HCl, and (c) redistilled water. The pH values of these solutions were determined at 23° C. with the glass electrode and found to be 2.05, 2.0, and 6.92, respectively.

Approximately 500 mg. of protein were employed for each solubility study. The protein was precipitated at pH 5.5 and washed with successive portions of each solvent until 2 successive solubilities were the same. The precipitate was then broken into a fine suspension and varying quantities distributed among tubes filled with the solvent. The tubes were rotated for 48 hours at 23° C., filtered and the filtrate analyzed for nitrogen by the Kjeldahl micro-procedure. The results of the solubility studies are shown in FIGURE 3. It will be seen that the solubility is independent of the amount of the saturating solid, from the first appearance of a solid phase. No solid phase appeared until the break in each of the curves in FIGURE 3, *i.e.*, before the slope became zero. Bioassays of the soluble and insoluble material did not show any physiological differences.

Sedimentation

A 2 per cent solution of crystalline prolactin at pH 8.0 was made by dissolving the protein with the aid of 0.1N sodium hydroxide and adjusting with 0.1N hydrochloric acid. This solution was examined in an analytical air-driven ultracentrifuge[18] arranged for optical study in the visible region by the Toepler schlieren method, as developed for the ultracentrifuge by Philpot.[19] The initial protein peak present at the start of the experiment sedimented at a uniform rate. A comparable run with highly purified, amorphous prolactin gave a similar diagram. In FIGURE 4 are reproduced the photographs obtained in a run with a crys-

[17] **Li, C. H., Lyons, W. R., & Evans, H. M.** Jour. Gen. Physiol. **24**: 303. 1941.
[18] **Beams, J. W., Linke, F. W., & Sommer, P.** Rev. Sci. Instr. **9**: 248. 1938.
[19] **Philpot, J. S. L.** Nature **141**: 283. 1938.

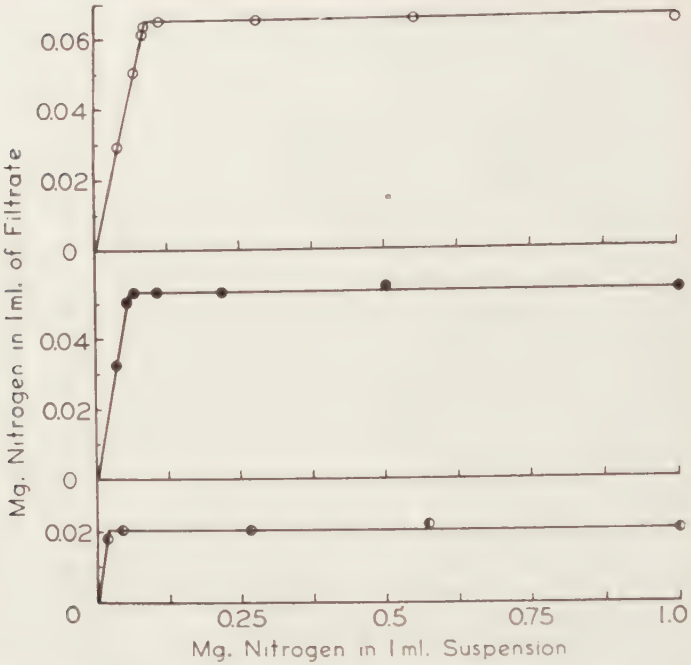

FIGURE 3. Solubility curves of crystalline prolactin in various solvents. Upper curve, solvent: 0.12 M NaCl solution in 0.01 M HCl, pH 2.05. Middle curve, solvent: 0.33 M NaCl solution in 0.01 M HCl, pH 2.0. Lower curve, solvent: redistilled water, pH 6.92. All measurements at 28° C.

talline preparation. The average value of the sedimentation constant, s_{20}, found for prolactin in several runs was approximately 2.8 S. If the prolactin molecule is tentatively assumed to be spherical, the sedimentation constant obtained indicates an approximate molecular weight[20] of the order of 35,000 for this protein hormone.

A highly purified, amorphous prolactin preparation (35 I.U./mg.) was sent to the laboratory of Professor J. W. Williams at the University of Wisconsin for examination in the Svedberg oil-driven ultracentrifuge. Several sedimentation and diffusion experiments have been completed.

[20] **Svedberg, T., & Pedersen, K. O.** "The Ultracentrifuge." Clarendon Press. Oxford. 1940. p. 406.

FIGURE 4. Sedimentation of crystalline prolactin. Photographs by Philpot schlieren method at 0, 30, 60, 90 and 120 min. intervals, reading from left to right. 45,000 r.p.m. (142,000 x g.

Professor Williams has written the following in a personal communication, "We have been able to finish a few sedimentation and diffusion experiments with your prolactin. The results we find are: $s_{20} = 2.65$ S, $D_{20} = 7.5 \times 10^{-7}$, and M = 32,000. I am inclined to think that the molecular weight value will eventually turn out to be a little larger than the figure we give. On sedimentation and diffusion the prolactin gave curves very much like those that one would expect when working with a simple homogeneous substance."

Li, Lyons, and Evans[16c] have recently determined the molecular weight of prolactin by osmotic pressure measurements and have also calculated the probable molecular weight on the basis of the cystine, arginine, tyrosine, tryptophane, and sulfur contents of the hormone. From these data the molecular weight of the lactogenic hormone was estimated to be approximately 25,000. It would appear evident on the basis of other information now available that this value is considerably too low.

The preliminary data obtained by Professor Williams and his colleagues will be extended when circumstances permit resumption of this work. It seems advisable to obtain as accurate physical-chemical constants as possible for any highly purified protein, and the physiological interest in prolactin adds importance to data obtained in a laboratory experienced in determining these constants. Grateful acknowledgment is made to Professor Williams and his colleagues for these preliminary figures.

ULTRAVIOLET ABSORPTION SPECTRUM

The ultraviolet absorption spectrum of prolactin has been measured by Dr. G. I. Lavin, of The Rockefeller Institute for Medical Research.[21] In FIGURE 5 are reproduced the absorption spectrum curves of crystalline prolactin and of a highly purified amorphous preparation. All the solutions were photographed at room temperature in aqueous medium at pH 7.4. The absorption curves were measured with the aid of a Spekker spectrophotometer and a small Hilger quartz spectrograph, with a tungsten steel spark as the light source.

The absorption curves obtained are typical of those found for a number of proteins, particularly those from animal tissue. The broad band with the maximum at about 2800 Å is to be attributed to the combined absorption of the amino acids, tryptophane, tyrosine, and phenylalanine, all of which have been demonstrated to be constituents of prolactin.

[21] **White, A., & Lavin, G. I.** Jour. Biol. Chem. **132**: 717. 1940.

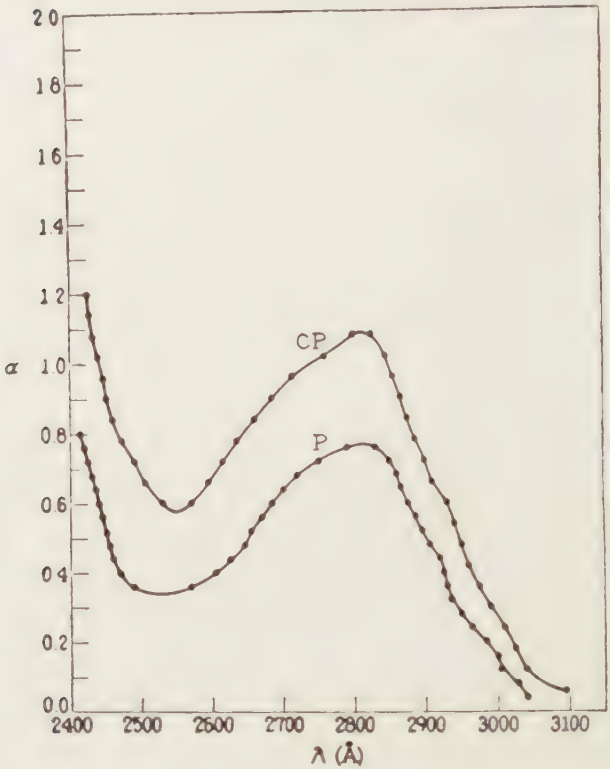

FIGURE 5. Absorption curves of solutions of prolactin. Curve (*P* represents crystalline prolactin; curve *P* was obtained with highly purified amorphous prolactin. pH 7.4. α (extinction coefficient) $\alpha = 1/cl \log I_0/I$, c = concentration (2.5 mg. per cc. in the case of curve *P*; 1.6 mg. per cc. in the case of curve (*P*), l = cell thickness in cm. (1 cm.), I_0 = initial intensity of light, I = intensity after passing through thickness l.

ISOELECTRIC POINT OF PROLACTIN

The isoelectric point of prolactin has been determined in two laboratories from mobility studies in the Tiselius apparatus. Values of pH 5.6,[14] pH 5.70,[15a] and pH 5.73[15b] have been reported. In view of the fact that the published values for the isoelectric point of prolactin have been based upon mobility studies in the Tiselius apparatus, it has seemed of interest to conduct a determination of the isoelectric point by the technique developed by Abramson and his colleagues.[23] This method is also an electrophoretic one, based upon direct measurement of the electrical mobility of microscopically visible quartz particles coated with an adsorbed layer of protein. The data obtained are plotted in FIGURE 6. It

[23] **Abramson, H. A.** "Electrokinetic Phenomena in Biology and Medicine." Chemical Catalog Co., Inc. New York. 1934.

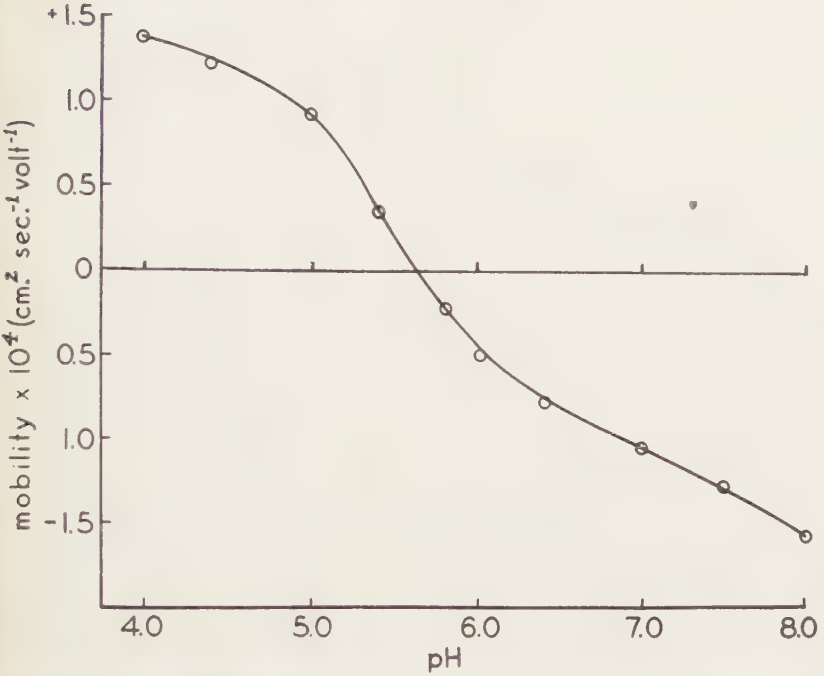

FIGURE 6. The electric mobility of quartz particles covered with prolactin in acetate and phosphate buffers of constant ionic strength (0.1). The isoelectric point is between pH 5.65 and pH 5.70.

will be seen that the isoelectric point of prolactin appears to be at pH 5.65, a value which is in good agreement with the previously published isoelectric point values obtained in the Tiselius apparatus.

ELEMENTARY ANALYSIS AND QUALITATIVE REACTIONS

Several preparations of crystalline and of highly purified, amorphous prolactin have been subjected to elemental microanalysis. These analyses have been conducted at intervals over the course of several years by Mr. J. F. Alicino on different preparations in order to check the constancy of composition and of analytical technique. The nitrogen values obtained by Mr. Alicino by means of the Dumas method have in all cases been checked in our laboratory by the Kjeldahl micro-procedure. In addition, a preparation of highly purified, amorphous prolactin obtained from Dr. Schwenk has also been quantitatively examined for its elemental composition. The data obtained are presented in TABLE 2, together with data obtained by Dr. Lyons and kindly provided by the latter investigator in a private communication. It will be seen that the elementary

composition of the prolactin preparations differs particularly in respect to its nitrogen content from the data reported several years ago.[11] The rather good analytical agreement among various preparations, taken together with the results of the bio-assays and certain unpublished physical-chemical and physiological results indicate that the prolactin products prepared in three different laboratories are probably the same protein.

TABLE 2
ELEMENTARY COMPOSITION OF VARIOUS PROLACTIN PREPARATIONS

Preparation	C	H	N	S	Ash
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Purified prolactin*	52.04	7.01	16.84	2.05	0.59
Crystalline prolactin*	51.81	6.81	16.49	2.03	0.50
Prolactin (Schwenk)*	51.10	7.00	16.61	1.98	0.72
Prolactin (Lyons)**	51.40	7.01	16.09	2.24	negligible
White, Catchpole and Long[11]*	51.11	6.76	14.38	1.77	
Li, Lyons and Evans[16c]				1.79	

* Each of the analytical values for these preparations represents the average of two determinations. All values in the table are calculated on an ash- and moisture-free basis.
** Personal communication from Dr. Lyons.

The crystalline and the purified products give the usual protein color tests (biuret, xanthoproteic, Millon's, Hopkins-Cole). The labile sulfur test is positive. Qualitative tests for phosphorus and for carbohydrate are negative. The nitroprusside test is negative. After reduction of a 1 per cent solution of prolactin with an equal volume of 5 per cent sodium cyanide, a weak but definitely positive nitroprusside reaction is obtained provided that cyanide reduction is permitted to proceed for approximately 2 hours at room temperature. Under comparable conditions, insulin was found to give a strongly positive sulfhydryl test within 5 minutes after the addition of the cyanide.

TYROSINE, TRYPTOPHANE, AND CYSTINE CONTENT OF PROLACTIN

Both crystalline and highly purified, amorphous prolactin preparations obtained from whole beef pituitary glands have been analyzed for tyrosine and tryptophane by the micro-method of Folin and Marenzi.[23] Cystine was determined by the method of Sullivan and Hess,[24] on an hydrolysate prepared by the HCl-formic acid procedure.[25] The results of these analyses are shown in TABLE 3 which, for comparison, also contains some analytical data from the literature.

[23] **Folin, O., & Marenzi, A. D.** Jour. Biol. Chem. **83**: 89. 1929.
[24] **Sullivan, M. X., & Hess, W. C.** Pub. Health Rep. U.S.P.H.S. suppl. **86**: 1930.
[25] **Miller, G. L., & du Vigneaud, V.** Jour. Biol. Chem. **118**: 101. 1937.

It will be seen that the values obtained for the tyrosine, tryptophane and cystine content of prolactin are in good agreement for the 2 preparations employed. The tyrosine value found confirms that reported for beef prolactin by Li, Lyons, and Evans.[16c,26] The tryptophane value obtained for prolactin is essentially the same (1.31 per cent) as that reported by Li, Lyons, and Evans,[26] who employed Lugg's[27] modification of the method of Folin and Ciocalteu.[28] More recently, however, these same workers have reinvestigated prolactin for its tryptophane content[16c] and report a value considerably higher (2.5 per cent) than that initially obtained.[26] The higher value is attributed to the fact that it was obtained by a glyoxylic acid method[29] which does not require hydrolysis of the protein, whereas the Lugg method originally employed for the determination of tryptophane involved an alkaline digestion which was believed to result in some destruction of the tryptophane.

TABLE 3

TYROSINE, TRYPTOPHANE, AND CYSTINE CONTENT OF BEEF PROLACTIN

Preparation	Tyrosine	Tryptophane	Cystine
	per cent	*per cent*	*per cent*
Purified amorphous prolactin	5.64	1.30	3.32
	5.50	1.27	3.41
Crystalline prolactin	5.42	1.34	3.34
	5.48	1.30	
Average*	5.51	1.30	3.36
Li, Lyons and Evans[26]	5.73	1.31	
Li, Lyons and Evans[16c]	5.7	2.5	
Fraenkel-Conrat[31]			3.0

* All values calculated on an ash- and moisture-free basis.

It is the opinion of the writer that Shaw and McFarlane,[29] who have studied the glyoxylic acid method in some detail, have not established that destruction of tryptophane occurs either under exactly the conditions of hydrolysis proposed by Folin and his colleagues[23,28] or in the procedure of Lugg.[27] Shaw and McFarlane have examined the effect of alkali and pressure on the stability of tryptophane in alkaline protein hydrolysates, but these are not the same hydrolytic conditions used in the Folin and Lugg procedures. Moreover, the glyoxylic acid method yields a tryptophane value for casein which is slightly lower than that

[26] **Li, C. H., Lyons, W. R., & Evans, H. M.** Jour. Biol. Chem. **136**: 709. 1940.
[27] **Lugg, J. W. H.** Biochem. Jour. **32**: 775. 1938.
[28] **Folin, O., & Ciocalteu, V.** Jour. Biol. Chem. **73**: 627. 1927.
[29] **Shaw, J. L. D., & McFarlane, W. D.** Canadian Jour. Research **B 16**: 361. 1938; Jour. Biol. Chem. **132**: 387. 1940.

obtained by Folin and his colleagues, notwithstanding the fact that the latter investigators employed alkaline hydrolysis of the protein.

According to Lugg, tryptophane estimations require correction for 3 per cent loss if stannite has been included in the alkaline hydrolysis, or 6 per cent when alkali alone has been used. Brand and Kassell,[30] in a careful and thorough study of the photometric determination of tryptophane, tyrosine, diiodotyrosine and thyroxine, based on the procedure developed by Lugg from the Folin-Ciocalteu method, have suggested that no correction factor is required for the tryptophane content of alkaline hydrolysates of proteins. In view of these above observations, the lower tryptophane values uniformly found in hydrolyzed as contrasted to unhydrolyzed proteins can hardly be attributed to the destruction of this amino acid during alkaline hydrolysis of the protein. Rather, the disagreement appears to be based on the type of material to which colorimetric procedures are applied.

The cystine value found for prolactin is slightly higher than that recently reported by Fraenkel-Conrat.[31] A cystine content of 3.36 per cent accounts for approximately 45 per cent of the total sulfur of prolactin.

EFFECT OF pH AND HEAT ON PROLACTIN ACTIVITY

Prolactin has been reported to be relatively thermostable when heated in a boiling water bath for 1 hour at pH 7.0 to 8.0, but less stable at other pH ranges. It is of course evident that many factors may influence the stability of protein hormones, *e.g.*, the purity of the product and thus the presence of extraneous proteins which may increase the resistance of the hormone to labilizing agents. In addition, protein and salt concentrations and the pH of the solutions examined may have a profound influence on the stability of a biocatalytically active protein.

Solutions containing 0.04 per cent of highly purified, amorphous prolactin (30 I.U. mg.) were heated in a boiling water bath for 15 and 30 minute periods at pH values from 1 to 13. The desired pH values were obtained with 0.01 N HCl and 0.01 N NaOH in suitable proportions. At the end of the heating period, each sample was cooled immediately to room temperature, neutralized to approximately pH 7.0, made to a suitable volume and bio-assayed by the "local" intradermal method at a total protein level of 1, 10, and 100 μg. The results are shown in TABLE 1 and graphically presented in FIGURE 7. Even though the minimum effective dose has not been determined in these bio-assays, it is evident from the data that under the conditions employed prolactin is quite

[30] **Brand, E., & Kassell, B.** Jour. Biol. Chem. **131**: 489. 1939.
[31] **Fraenkel-Conrat, H.** Jour. Biol. Chem. **142**: 119. 1942.

TABLE 4

EFFECT OF pH AND HEAT ON PROLACTIN ACTIVITY

pH	Period of heating	Total dose injected for bio-assay		
		μg.*		
		1	10	100
	min.			
1	15	+	+	+
	30	0	+	+
3	15	+	+	+
	30	0	0	+
7	15	+	+	+
	30	0	0	+
9	15	+	+	+
	30	0	0	+
11	15	0	+	+
	30	0	0	+
13	15	0	0	+
	30	0	0	0

* + = positive response—active; 0 = negative response—inactive.

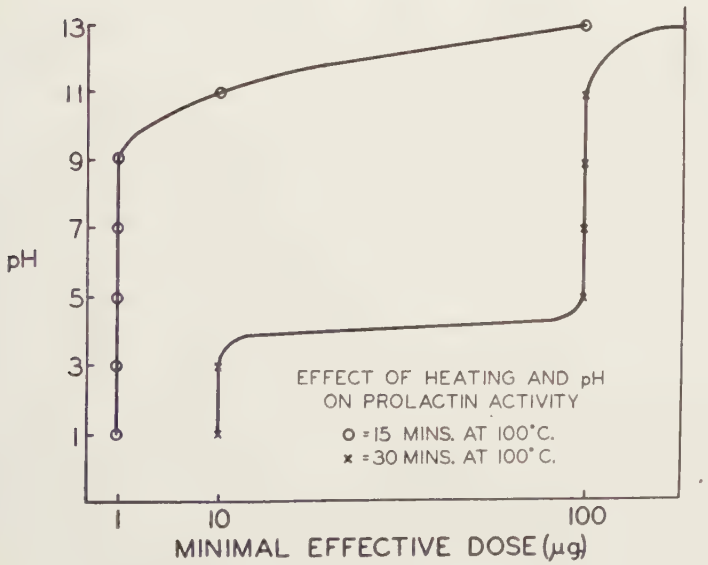

FIGURE 7. Effect of pH and heat on prolactin activity.

stable to heat in 0.04 per cent solutions of pH 1 to 9 when the heating is carried out for 15 minutes in a boiling water bath. At higher pH values there is a considerable loss of biological potency. With a 30 minute period of heating, there is a definite destruction of prolactin activity at all pH values, the effect again appearing to be most marked at pH 13. It appears, therefore, that prolactin may be classed as a heat-labile substance; this is in harmony with the protein nature of the hormone. The destruction of the hormone observed in the present experiments might find explanation in the splitting of labile sulfur from the hormone and in a significant degree of hydrolysis of the protein.

HYDROLYSIS OF PROLACTIN

Hydrolysis by Acid

Prolactin boiled for 18 hours with 20 per cent hydrochloric acid produces a hydrolysate which, after filtration and neutralization, gives no evidence of biological activity even though assays are conducted at a dose level approximately 400 times (based on the original protein concentration) that required to produce a positive response by the unhydrolyzed prolactin.

Digestion by Pepsin and by Trypsin

The prolactin used in the digestion experiments was active intradermally at a dose level of 0.5 μg. For the pepsin study, a 1:10,000 granular pepsin of the Wilson Laboratories was used. Digestion was conducted in 0.1 N HCl at 37° C. The ratio of enzmye to prolactin was 1 : 250. After mixing the enzyme and prolactin solutions, samples were taken immediately and at 1, 2, and 3 hours of digestion time. Peptic action was stopped by making the samples slightly alkaline (pH 7.5) with 0.1 N NaOH and permitting 15 minutes to elapse.

In the trypsin experiments, a commercial trypsin preparation (Fairchild) was employed. Digestion was studied at pH 7.8 in phosphate buffer. The ratio of trypsin to prolactin was 1:50. Samples were taken at zero time, and at $^1/_2$, 1, 2 and $3^1/_2$ hour periods. In order to stop further tryptic action in the samples taken for analysis, each was immersed in a boiling water bath for 1 minute, cooled rapidly and diluted to a convenient volume for assay. Bio-assays were conducted by the intradermal technique. The rate of protein digestion was followed as described by Northrop,[32] analyses being made for nonprotein nitrogen

[32] **Northrop, J. H.** "Crystalline Enzymes; the Chemistry of Pepsin, Trypsin, and Bacteriophage." Columbia Univ. Press, New York. 1939.

in filtrates obtained after precipitation of each 0.5 ml. sample with an equal volume of 10 per cent trichloroacetic acid.

The rate of hydrolysis of prolactin by pepsin and by trypsin is shown in FIGURE 8, and the results of the bio-assays are presented in TABLES

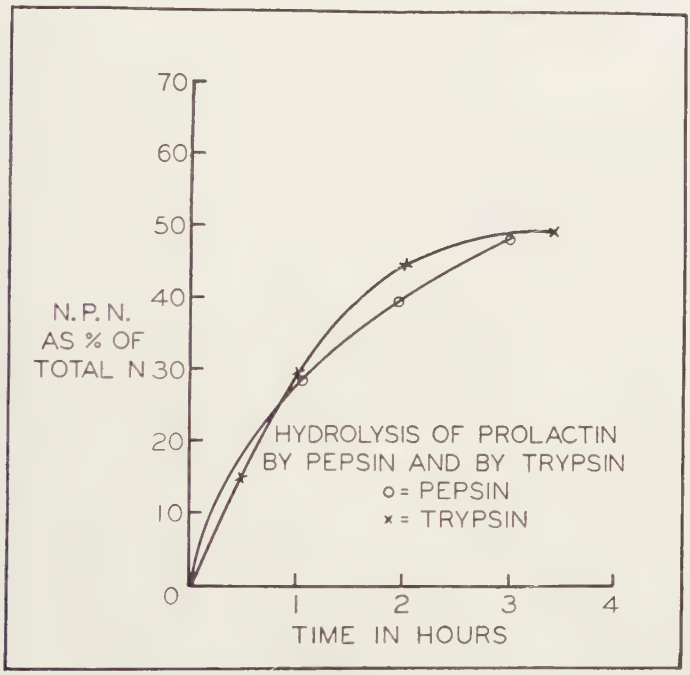

FIGURE 8.

5 and 6. It will be seen that in the case of digestion with either pepsin or trypsin, approximately half of the original protein nitrogen is no longer precipitable by trichloroacetic acid after a 3-hour period of digestion. However, even though at the end of a 2-hour period there still remained in solution as much as 50 to 60 per cent of nitrogen which could be precipitated by trichloroacetic acid, the bio-assays show that this material was biologically inactive, even when given at dose levels from 100 to 120 times the amount necessary to elicit a positive prolactin response with the original prolactin preparation. In other words, under the conditions of these enzyme studies, destruction of physiological activity occurs before there has been a hydrolysis of the protein into fragments which are no longer precipitable by trichloroacetic acid.

TABLE 5

RATE OF DIGESTION OF PROLACTIN BY PEPSIN AS CORRELATED WITH ALTERATIONS IN BIOLOGICAL ACTIVITY OF THE HORMONE

Time	Per cent of original protein digested (non-precipitated by 10 per cent trichloroacetic acid solution)	Bio-assay. Total dose level μg.†		
hrs.				
0	0	1*	10*	100*
1	29	0.7	7*	70*
2	40	0.6	6	60
3	48	0.5	5	50

† All dosages, with the exception of those at 0 time, were calculated from nitrogen analysis.
* Active, *i.e.*, positive prolactin response. All other assays were negative at the dose levels indicated.

TABLE 6

RATE OF DIGESTION OF PROLACTIN BY TRYPSIN AS CORRELATED WITH ALTERATIONS IN BIOLOGICAL ACTIVITY OF THE HORMONE

Time	Per cent of original protein digested (non-precipitated by 10 per cent trichloroacetic acid solution)	Bio-assay. Total dose level μg.†		
hrs.				
0	0	1*	10*	100*
0.5	15	0.8*	8*	80*
1.0	30	0.7*	7*	70*
2.0	47	0.5	5	50
3.5	49	0.5	5	50

† All dosages, with the exception of those at 0 time, were calculated from nitrogen analysis.
* Active, *i.e.*, positive prolactin response. All other assays were negative at the dose levels indicated.

The data obtained in the study of the enzymatic digestion of prolactin are similar to those of Fisher and Scott[33] on the peptic hydrolysis of insulin. These investigators observed that insulin activity was rapidly destroyed by pepsin and, indeed, the hypoglycemic potency decreased more rapidly than the rate of hydrolysis of the protein.

OTHER TYPES OF INACTIVATION

The preparation of a highly purified, biocatalytically active protein is generally soon followed by efforts to determine whether particular groups or groupings in the protein molecule are essential for the activity which it manifests. It is to be hoped that studies of this type will, to some extent by themselves, and perhaps even more when correlated with similar experiments on other proteins, begin to lead to some understanding regarding the fundamental underlying mechanism of imparting to a particular protein a certain type of catalytic activity. The mechanism of action of certain proteins which do not contain the usually well recog-

[33] **Fisher, A. M., & Scott, D. A.** Jour. Biol. Chem. **106**: 289. 1934.

nized prosthetic groups is one of the important unsolved problems of protein chemistry.

Inactivation with Ketene

The necessity of primary amino groups for the specific activity of the lactogenic hormone has been examined by investigators in the laboratory of Evans in California. In a preliminary note,[34] the acetylation of prolactin with ketene at 20° C. for 5 minutes is claimed to result in a 100 per cent acetylation of the amino groups of the hormone, accompanied by a complete physiological inactivation. These data are shown in TABLE 7

TABLE 7

ACETYLATION OF PROLACTIN BY KETENE

Type of preparation	Amino groups acetylated	Dose	Crop-sac reaction
	per cent	*mg.*	
Untreated	0	1.0	Pronounced
Acetylated at 0° for 5 min.	30	1.0	Negative to minimal
Acetylated at 20° for 5 min.	100	1.0	Negative
		4.0	Negative

and are taken from the communication of Li, Simpson and Evans.[34] It should be pointed out that in this study it has not been established that substitution of groups other than free amino groups has not occurred. This should be demonstrated for each protein studied, inasmuch as rates of substitution reactions with ketene for one protein are not necessarily valid for interpretation of data obtained in ketene experiments with other proteins.

It is of interest to compare these data for prolactin with the behavior of other well defined, biologically active proteins when treated with ketene. Whereas the practically complete acetylation of the free amino groups of pepsin,[35] insulin,[36] human chorionic gonadotropin[37] and, to a considerable degree, tobacco mosaic virus[38] by ketene does not diminish the biological activity of these proteins, diphtheria toxin,[39] the pituitary follicle cell-stimulating, interstitial-cell-stimulating[37] and lactogenic hormones,[34] and the gonadotropic hormone in pregnant mare serum[37] appear to require free amino groups for their activity.

[34] **Li, C. H., Simpson, M. E., & Evans, H. M.** Science **90**: 140. 1939.
[35] **Herriott, R. M., & Northrop, J. H.** Jour. Gen. Physiol. **18**: 35. 1934.
[36] **Stern, K. G., & White, A.** Jour. Biol. Chem. **122**: 371. 1937–38.
[37] **Li, C. H., Simpson, M. E., & Evans, H. M.** Jour. Biol. Chem. **131**: 259. 1939.
[38] **Miller, G. L., & Stanley, W. M.** Jour. Biol. Chem. **141**: 905. 1941.
[39] **Pappenheimer, A. M., Jr.** Jour. Biol. Chem. **125**: 201. 1938.

Inactivation with Nitrous Acid

Further evidence that the primary amino groups of the lactogenic hormone are essential for its specific activity has been claimed from a study of its behavior toward nitrous acid.[40] Treatment of prolactin with nitrous acid for 30 minutes at zero degrees or at room temperature resulted in complete inactivation of the hormone. No evidence establishing the mode of action of nitrous acid on prolactin is offered. The oxidizing action of the reagent must be considered in making interpretations of changes in biocatalytic activity after treatment with nitrous acid.

Inactivation with Phenyl Isocyanate

In a preliminary communication, Bottomley and Folley[41] reported the preparation of a phenyl ureido derivative of prolactin by treatment of the protein with phenyl isocyanate at 0° C. and pH 8.0. A loss of approximately 87 per cent of the biological activity was observed. The data are taken as confirmation of the suggestion that the crop-stimulating activity of prolactin depends on the presence in the molecule of free amino groups. It would seem advisable, however, in experiments involving the treatment of proteins with phenyl isocyanate, to demonstrate clearly by analysis that this reagent reacts *only* with the free amino groups of the particular protein being studied. Miller and Stanley[25] have recently observed a marked reaction between phenyl isocyanate and the phenolic groups in the tobacco mosaic virus protein. The reported inactivation of both prolactin[41] and of insulin[42] by phenyl isocyanate is open to other interpretations, particularly in view of the work of Miller and Stanley, and the demonstration, by the use of ketene, that the amino groups of insulin are not essential for the hypoglycemic action of the latter protein.[36]

Inactivation with Iodine

The reaction of iodine with lactogenic hormone has been studied in some detail by Li, Lyons, and Evans.[40] Iodination in phosphate buffer pH 7.0 at 20° C. for 1 hour resulted in an introduction of iodine into the phenolic residues of the tyrosine molecules of prolactin, and a complete inactivation of the hormone.

Inactivation with Thiol Compounds

Fraenkel-Conrat, Simpson and Evans[43] have recently described results obtained in a study of the effect of thiol compounds on the activity of the

[40] **Li, C. H., Lyons, W. R., Simpson, M. E., & Evans, H. M.** Science **90**: 376. 1939.
[41] **Bottomley, A. C., & Folley, S. J.** Nature **145**: 304. 1939.
[42] **Gaunt, W. E., & Wormall, A.,** Biochem. Jour. **30**: 1915. 1936.
[43] **Li, C. H., Lyons, W. R., & Evans, H. M.** Jour. Biol. Chem. **139**: 43. 1941.
[44] **Fraenkel-Conrat, H., Simpson, M. E., & Evans, H. M.** Jour. Biol. Chem. **142**: 107. 1942.

lactogenic hormone. Treatment of a solution of prolactin with a 40-fold concentration of cysteine transforms the hormone into an insoluble protein which, when redissolved under conditions which prevent autoxidation, is as biologically active as the untreated hormone. When a 200-fold quantity of cysteine is employed to reduce prolactin, inactivation occurs. Thioglycolic acid is approximately 50 times more effective than cysteine in causing inactivation of the lactogenic hormone.

COMMENT AND SUMMARY

Prolactin is the first anterior pituitary hormone to have been isolated in pure form. The homogeneous nature of the preparations obtained is indicated from Tiselius electrophoresis experiments, solubility studies, and ultracentrifugal analysis. All of the evidence available at the present time indicates the protein nature of the hormone; there is no indication of the presence in the molecule of any of the common types of prosthetic groupings.

The protein hormone has a molecular weight of approximately 32,000 to 35,000 and an isoelectric point at pH 5.65. Some data for beef prolaction are shown in TABLE 8. Prolactin may be classified as a heat-

TABLE 8
SOME DATA FOR BEEF PROLACTIN

Physiological activity	35 I. U./mg.
Carbon	51.50 per cent
Hydrogen	6.92 " "
Nitrogen	16.50 " "
Sulfur	2.00 " "
Tyrosine	5.7 " "
Tryptophane	1.3 " "
Cystine	3.4 " "
S_{20}	2.7 S
D_{20}	7.5×10^{-7}
Molecular weight	32,000–35,000
Isoelectric point	pH 5.65
Ultraviolet absorption maximum	2800 Å

labile protein. Furthermore, reagents which produce even a mild degree of hydrolysis of the protein molecule cause a profound loss in the hormonal activity. From studies directed toward an assay of the physiologically important functional groups in the prolactin molecule, it appears that substitution of the free amino groups or of the tyrosine phenolic groups of the protein results in a marked decline in, and usually a complete loss of, lactogenic potency. Reduction of prolactin with an excess of cysteine or of thioglycolic acid also destroys biological activity.

The preparation of a pure hormone of the anterior pituitary gland will be of considerable aid in elucidating the complex physiology of this endocrine gland, and the rate of progress in this direction will be greatly accelerated as each additional anterior pituitary secretion is made available in a purified and homogeneous state. It is naturally of great interest to determine whether the prolactin preparations now available exhibit any other type of physiological activity which has been attributed to anterior pituitary extracts. Preliminary studies of this type have been conducted; the results are indicated in TABLE 9.

TABLE 9
BIO-ASSAYS OF PROLACTIN

Assay	Method	Result
Growth	Hypophysectomized rat	Negative
Thyrotropic	Day-old chick	"
Diabetogenic	Partially depancreatized rat	"
Adrenotropic	21-day-old rat	"
Gonadotropic	21-day-old mouse	"

It should be stressed that these are preliminary data and in each assay an amount of prolactin was administered which was approximately 100 times the amount of the protein of a whole beef pituitary gland saline extract (in terms of nitrogen times 6.25) which would yield a positive response in the test employed. These data are obviously not final. It is quite possible that similar tests at higher dose levels, or in other species, may indicate a multiple type of physiological activity for prolactin. The overlapping physiological behavior seen among the steroid hormones at high dose levels and under specially defined conditions of assay should be kept in mind. Indeed, data are already available indicating physiological activity for prolactin other than crop proliferation and initiation of lactation. Evans and his colleagues have recently reported evidence for two other types of physiological responses induced by this hormone. By means of the traumatic placentoma test in the rat, it has been demonstrated[39] that lactogenic hormone stimulates the production of progesterone by normally occurring or artificially induced lutein tissue. This interesting confirmation with purified prolaction of earlier suggestions that the lactogenic hormone is important in activation of the *corpus luteum* adds another example to the list of endocrine interrelationships. Equally striking is the recent claim from Evans' laboratory[40] that the lactogenic hormone increases the insulin content of the pancreas in nor-

[39] **Evans, H. M., Simpson, M. E., & Lyons, W. R.** Proc. Soc. Exp. Biol. Med. **46**: 586. 1941.
[40] **Fraenkel-Conrat, H., Herring, V. V., Simpson, M. E., & Evans, H. M.** Am. Jour. Physiol. **135**: 404. 1942.

mal and in hypophysectomized rats. The data presented, however, do not appear significant when evaluated critically and require elaboration and confirmation by other investigators. This is particularly true in view of the fact that prolactin has also been reported to decrease pancreatic insulin.[47] The exact effect of prolactin on the insulin content of the pancreas should be determined by the use of experimental conditions rigidly established by Best and his colleagues at the University of Toronto.

These newer developments in the physiology of the lactogenic hormone, together with recent results[48] obtained in collaboration with Dr. W. U. Gardner and discussed in the next section of this paper, are further emphasis of the fact that the nature of the physiological response manifested by a particular endocrine product may often be determined by the type of test animal and the dosage employed.

Section II: Mammogen

INTRODUCTION

The name, mammogen, was proposed in 1938 by Turner and his coworkers to describe the hormonal complex in the anterior pituitary which they believe is concerned with growth and development of the mammary gland. From experimental evidence at hand, the existence of two mammogen factors was postulated: one a duct growth factor, designated Mammogen I; and the other, named Mammogen II, believed to be responsible for completion of mammary development, causing growth of the mammary lobule-alveolar system.

EVIDENCE FOR THE EXISTENCE OF MAMMOGEN

The concept that the anterior pituitary gland exerts an important influence on the growth and development of the mammary gland has been generally accepted since 1936, when Reece, Turner, and Hill[49] reported that the mammary glands of completely hypophysectomized rats did not respond to the administration of ovarian hormones; a role of the anterior pituitary in the process of proliferation of mammary gland tissue seemed clearly indicated. This was further substantiated by the effectiveness of hypophyseal replacement therapy in hypophysectomized animals. The administration of crude anterior pituitary extracts, either alone or

47 **Funk, C., Chamelin, I. M., Wagreich, H., & Harrow, B.** Science **94**: 260. 1941.
48 **Gardner, W. U., & White, A.** Proc. Soc. Exp. Biol. Med. **48**: 590. 1941.
49 **Reece, R. P., Turner, C. W., & Hill, R. T.** Proc. Soc. Exp. Biol. Med. **34**: 204. 1936.

with ovarian substance, to hypophysectomized animals of several species permitted normal growth and development of mammary glands.

Without presenting a detailed survey of the publications dealing with the problem of the existence of a pituitary mammogenic hormone, the conclusion is warranted that crude anterior pituitary extracts exert a profound influence on mammary growth.[50] The first experiments revealing the lactogenic activity of hypophyseal extracts were inadequate for the demonstration of mammary growth. Later experiments suggested a proliferation of the mammary glands of immature ovariectomized animals receiving pituitary extracts. This growth occurs in ovariectomized, in male or in hypophysectomized mice, or in rats following implantation of hypophyseal tissue or injection of hypophyseal extracts. Some investigators have associated this mammogenic activity with the protein components of such extracts and others with the lipid-soluble material of pituitary tissue.

Accepting the evidence that anterior pituitary gland tissue extracts contain a factor or factors which influence mammary development, and which have been termed mammogen by Turner and his colleagues, it is the purpose of the remainder of this discussion to consider three aspects of this problem: (1) the available information regarding the chemical nature of mammogen, (2) the relationship of mammogen to known hormones, and (3) the validity of the data supporting the mammogen hypothesis. Before passing to these topics, it may be of interest to mention briefly the methods devised for the assay of mammogen and mammary-stimulating materials.

METHODS OF ASSAY OF MAMMOGEN AND MAMMARY STIMULATING MATERIALS

In their studies of mammogen, Turner and his colleagues have employed the mouse as the assay animal. The activity of the anterior pituitary duct-growth factor (Mammogen I) has been measured by its effect on the mammary duct system of male mice. The biological assay of the anterior pituitary lobule-alveolar growth factor (Mammogen II) has been conducted in castrate, virgin female mice given small amounts of estrone which appears to enhance the activity of the pituitary lobule-alveolar growth factor. Other investigators have preferred to use hypophysectomized animals in order to eliminate the influence of the test subject's own pituitary and also to permit the evaluation of various extracts in terms of effectiveness as replacement therapy.

[50] **Turner, C.W.** in "Sex and Internal Secretions," 2nd edit. E. Allen, Ed. Williams and Wilkins Co. Baltimore. 1939 p. 740. (Complete review of literature on the mammary gland.

CHEMICAL PROPERTIES OF MAMMOGEN

Preparations Soluble in Lipid Solvents

The first report of the full development of the mammary glands in experimental animals injected with anterior pituitary preparations was made in 1930 by Corner,[51] who employed an alkaline extract of sheep anterior pituitary glands. He stated that ether extraction of this aqueous solution appeared to remove the mammary growth-promoting activity. The ether-soluble fractions, however, exhibited variable physiological potency; the active principle was rapidly destroyed on heating for 1 hour at 90° C. and appeared to deteriorate rapidly on storage.

The bulk of the evidence that the mammogenic factors in the anterior pituitary are soluble in lipid solvents has been derived from the investigations of Turner and his colleagues. Following their development of a suitable assay technique for the duct growth component of mammogen, Lewis and Turner[52] studied the chemical characteristics of the factor and concluded that this hormone is soluble in lipid solvents and is distinct from other known pituitary hormones. The evidence on which this conclusion was based may be briefly summarized (potency refers to activity in promoting duct growth in the rudimentary mammary glands of young male mice):

(1) Desiccation of fresh anterior pituitary lobe tissue from pregnant cattle with acetone and ether results in a 62 per cent loss of potency.

(2) The extraction of acetone-dried pituitary glands by a procedure (60 per cent alcohol at pH 9.0 to 10.0) which removed most of the lactogenic, thyrotropic, carbohydrate metabolism, and gonadotropic potency of fresh pituitary tissue[53] leaves an insoluble residue which is active. It should be pointed out, however, that precipitates prepared from the initial extracts by the addition of 3 volumes of 95 per cent alcohol at pH 5.7 also showed, after drying with alcohol and ether, a definite but smaller degree of potency.

(3) Residues obtained by evaporation of the 87 per cent alcohol solution used to precipitate the initial extract gave strongly positive results.

(4) Similar experiments with fresh, anterior pituitary tissue also yielded alcoholic solutions, after precipitation of the protein hormones in the usual manner, which on evaporation at low temperature *in vacuo* left highly potent residues. However, direct extraction of ground an-

[51] **Corner, G. W.** Am. Jour. Physiol. **95**: 43. 1930.
[52] **Lewis, A. A., & Turner, C. W.** Mo. Agr. Exp. Sta. Res. Bull. No. 310. 1939.
[53] **Bergman, A. J., Houchin, O. B., & Turner, C. W.** Endocrinology **24**: 547. 1939.

terior pituitary tissue with several volumes of 95 per cent alcohol did not yield active products.

(5) Extraction with several volumes of ether-alcohol mixture (1:3) at 50° C. was very efficient in removing mammogen. Removal of the solvent at reduced pressure yielded an active residue. The latter was fractionated by ether extraction at room temperature. The ether-soluble fraction is highly active; the ether-insoluble material is inactive. The best product reported to the present time was obtained in this manner. This preparation gave a positive response in 11 of a group of 14 mice at a dosage of 0.25 mg. per mouse.

Evidence that two distinct mammogen factors are concerned with growth and development of the mammary gland has also been reported by Turner and his colleagues. Fresh anterior pituitary tissue containing mammogen produced both duct development and lobule hyperplasia, whereas the lipid extracts caused only duct development.[52,54]

There have as yet been little data available regarding the chemical nature of mammogen, other than the claim of solubility in lipid solvents at pH ranges in which pituitary protein hormones are precipitable. This behavior is said to differentiate mammogen from other known anterior pituitary hormones. Lewis and Turner[52] reported that mammogen is quite unstable to heat and possibly to oxidation. The heat lability of mammogen has been stressed as a property which distinguishes this hormone from estrogenic substances. The active lipid extracts deteriorated rapidly if not kept at low temperatures.

ANTERIOR PITUITARY PROTEIN HORMONES IN RELATION TO MAMMARY GROWTH

Before discussing further the suggestions of Turner and his colleagues that the anterior pituitary secretes a hormonal complex, mammogen, which is distinct from other known anterior pituitary hormones, it may be advisable first to indicate the established roles of certain anterior pituitary protein hormones in the growth of the mammary gland. Furthermore, experiments will be described in which growth and development of the mammary gland in hypophysectomized animals are produced with already known and highly purified hormonal preparations. In view of these observations, it may not be necessary to postulate a new anterior pituitary complex, mammogen, as a regulatory factor required for mammary gland growth, and it then becomes permissible to re-evaluate previous experimental results in the light of the newer data.

The anterior pituitary hormones whose chemical nature has been

[54] **Mixner, J. P., Lewis, A. A., & Turner, C. W.** Endocrinology **28**: 888. 1940.

studied in more or less detail appear to be proteins. Although a number of negative results have been reported in early investigations, it is now clearly established that crude aqueous extracts of anterior pituitary tissue contain a factor, or factors, which cause mammary gland development in a wide variety of different species, both in normal and in hypophysectomized test animals. Although Corner[51] and later Turner and his colleagues[52] have, as already discussed, associated the pituitary factor with material soluble in lipid solvents, some investigators have also reported positive results with crude anterior pituitary protein fractions. The activity of these products is generally enhanced by the simultaneous administration of estrogen, although the latter is not always essential when crude pituitary extracts are employed. Asdell and his colleagues[55] reported that extracts of anterior pituitary gland of the sheep prepared by extraction with sodium hydroxide were found to cause mammary growth and secretion in the virgin female ovariectomized rabbit. Acid extracts were without effect on mammary growth, but induced lactation in the dry parous ovariectomized rabbit. This action was due to the prolactin in the acid extract. These data were interpreted as suggesting that the mammary growth and secretion effects are due to separate substances.

Greep and Stavely,[56] in a recent effort to confirm the presence of a lipid-soluble mammogen in the pituitary gland, assayed the warm (50° C.) alcohol-ether soluble material from both desiccated and fresh pituitary glands. Whereas the desiccated and powdered pituitaries, injected in suspension in saline, produced unquestionable mammary growth in doses equivalent to 50 mg. of fresh gland, the administration of lipid-soluble residues in doses equivalent to as much as 3200 mg. of fresh gland failed to induce mammary development. This was true of both types of alcohol-ether extracts, *i.e.*, whether made from desiccated pituitary tissue or from the fresh gland. On the other hand, Greep and Stavely reported marked duct growth in the test animals given the alcohol-ether-insoluble tissue residue in doses equivalent to 200 mg. of fresh gland.

The observations of Greep and Stavely that mammary growth factor is demonstrable in the protein fraction of pituitary tissue, and not evident in lipid extracts, make advisable a re-evaluation of the role which the known pituitary hormones may play in mammary gland growth. Lyons and Catchpole[57] in 1933 stated that a mammary gland in a virgin female

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55 **Asdell, S. A., Brooks, H. J., Salisbury, G. W., & Seidenstein, H. R.** Cornell Univ. Agr. Exp. Sta. Mem. **198**. 1936.
56 **Greep, R. O., & Stavely, H. E.** Endocrinology **29**: 18. 1941.
57 **Lyons, W. R., & Catchpole, H. R.** Proc. Soc. Exp. Biol. Med. **31**: 299. 1933.

rabbit, normally matured and then castrated, will respond by growth of ducts and alveoli and lactation to administration of pituitary lactogenic extract. It is interesting to note, in view of later developments, that these investigators stated their belief that if significant amounts of ovarian hormones remained in the body after castration, these hormones could act only by synergistic stimulation with the lactogenic hormone. In 1936, Lyons[58] reiterated his view that lactogenic hormone could induce alveolar hyperplasia by demonstrating secretion and alveolar proliferation following injection of prolactin into young male rabbits previously treated for a period of 3 weeks with daily injections of estrogen. In a footnote appearing in a recent publication of Evans, Simpson and Lyons,[59] however, it is stated that as much as 10 mg. of purified lactogenic hormone have been injected into both normal mature and hypophysectomized female rats without stimulating the proliferation of mammary lobules. Lyons and his colleagues[59] have corrected the observation in the above referred footnote and report that the injection of prolactin into young, normal female rats produces excellent mammary development. This mammary gland growth is attributed to the combined action of estrin and progestin.

In 1937, Gomez and Turner[60] reported their inability to demonstrate the growth of the duct system of the mammary glands of completely hypophysectomized rats and guinea pigs treated with purified thyrotropic, adrenotropic, or lactogenic hormones alone and in combination with estrogen and progesterone. Desiccated whole sheep pituitaries were also without effect under similar experimental conditions. In view of the recent demonstration by Greep and Stavely[36] that desiccated beef pituitaries produce definite mammary growth, and the observations of Gardner[61] that the mammary glands of hypophysectomized male mice showed development when estrogen and progesterone were injected, the negative findings of Gomez and Turner with similar preparations make necessary a re-examination of the effects of individual pituitary protein hormones which have been reported to be without stimulative action on the mammary gland.

Moreover, the striking demonstrations of the local action of estrogens on mammary development in a variety of species throw further doubt on the absolute necessity of the pituitary in the experimental development of the mammary glands and rather emphasize the observations on estrogen and progesterone, which Gomez and Turner claimed were with-

[58] **Lyons, W. R.** Anat. Rec. **64**: 31, suppl. 1936.
[59] **Lyons, W. R., Simpson, M. E., & Evans, H. M.** Proc. Soc. Exp. Biol. Med. **48**: 634. 1941.
[60] **Gomez, E. T., & Turner, C. W.** Mo. Agr. Exp. Sta. Res. Bull. No. 259. 1937.
[61] **Gardner, W. U.** Proc. Soc. Exp. Biol. Med. **45**: 835. 1940.

out effect on mammary proliferation. For example, Gardner and Chamberlin[62] in a recent study with male mice have reported that estrone in alcoholic solution when applied to one or more areas of the non-epilated skin produces growth of only those glands near the site of application of the estrone. Such experiments indicate that the response of the mammary tissue *in vivo* is directly stimulated through the action of estrogen. If the response of the glands to estrogen were necessarily mediated by a pituitary factor, all of the glands should have responded as well as did those to which the hormone was applied. In the hypophysectomized rat,[63] however, treatment with estrogen on the skin over the mammary gland is without effect, although in the normal or partially hypophysectomized rat, new growth is stimulated. These experiments are interpreted as further evidence that a pituitary mammogen is essential for steroid hormone effects on the mammary glands of hypophysectomized rats. It may be granted that a known pituitary factor affects mammary gland growth, without postulating a new mammogen complex. This is particularly true in view of the role of prolactin in stimulating mammary development.[48] These experiments will be considered later. The failure of the hypophysectomized rat to respond to combinations of desoxycorticosterone and estrogen[63] is in contrast to the behavior of the hypophysectomized mouse which exhibits definite mammary growth following treatment with these hormones.[61] It would have been of considerable interest if Leonard and Reece[63] had administered progesterone together with estrogen to their hypophysectomized rats. This combination of steroid hormones is effective in the hypophysectomized mouse, and had similar results been obtained with the rat, the ineffectiveness of local application to mammary tissue in hypophysectomized rats might then be attributed to the absence of prolactin and the capacity of the latter hormone to stimulate the production of progesterone.[45] It may also be pointed out that mammary development has been reported[55] in hypophysectomized rabbits injected with estrogen and progesterone. This species, therefore, behaves in a manner similar to the mouse in its response to a combination of these two steroid hormones.

Reece and Leonard[64] have recently published data contributing to the subject of the effects of pituitary protein hormones on the mammary gland. Hypophysectomized rats given estrogen and gonadotropic hormone prepared either from pregnancy urine or from gland sources exhibited no mammary proliferation. On the other hand, the mammary glands of experimental animals receiving a growth hormone preparation

62 **Gardner, W. U., & Chamberlin, T. L.** Yale Jour. Biol. Med. **13**: 462. 1941.
63 **Leonard, S. L., & Reece, R. P.** Endocrinology **30**: 32. 1942.
64 **Reece, R. P., & Leonard, S. L.** Endocrinology **29**: 297. 1941.

plus estrogen showed a stimulated growth of the duct system. It is interesting to note that the growth hormone preparation alone produced slight mammary growth. The growth hormone used contained thyrotropic hormone and probably small amounts of adrenocorticotropic substance. Gonadotropic hormone was also present in the growth preparation but the effect of the former can be disregarded in view of the negative findings with purified gonadotropic substances. The results obtained by Reece and Leonard were interpreted by those authors in terms of the effect of the mammogenic factor contained in the growth hormone preparation.

In collaboration with Dr. W. U. Gardner, of the Department of Anatomy of the Yale University School of Medicine, it has been possible recently to examine the influence of certain purified pituitary protein hormones on mammary growth in hypophysectomized male mice receiving estrogen. Gardner[64] has demonstrated that the mammary glands of hypophysectomized male mice may be developed slightly by the administration of estradiol propionate, progesterone, or desoxycorticosterone. A more extensive and more rapid proliferation of the mammary ducts of hypophysectomized mice occurred when desoxycorticosterone acetate or progesterone was injected together with the estrogen. Highly purified lactogenic hormone, prepared in our laboratory and shown to be homogeneous by solubility studies, electrophoretic behavior, and ultracentrifugal analysis was available, and this preparation has been tested for mammary-growth stimulating effects in hypophysectomized male mice receiving estrogen. Several other prolactin preparations were also studied in the following manner. Hybrid male mice from 4 to 8 weeks of age were hypophysectomized by means of the procedure described by Thomas.[65] The mice were maintained on a stock diet of Fox Chow and water. Completeness of operation was checked by examination of serial sections of the sella. From 3 to 47 days after operation, injections of lactogenic hormone with or without estrogen were started; the prolactin was administered intraperitoneally daily and the estrogen[66] subcutaneously every other day. On the 10th to 13th day the animals were killed and the mammary glands studied from stained and dissected preparations. Untreated hypophysectomized mice served as controls. Preliminary experiments have also been conducted with other types of extracts and hormonal preparations.

Some of the data obtained have already been published.[48] Since the time of the latter publication, experiments have been extended and a

[65] **Thomas, F.** Endocrinology **22**: 99. 1938.

[66] The estradiol dipropionate and 2 preparations of prolactin were generously supplied by the Schering Corporation through the courtesy of Dr. E. Schwenk.

summary of the results are shown in TABLE 10. Five mice received only a commercial preparation of prolactin (SC) which contained 12.5 I.U. per mg. A slight mammary growth occurred in 3 of these animals. Two of the latter were caged with animals receiving estrogen and may have acquired enough of the latter hormone by contact to elicit some mammary response. A moderately extensive mammary growth occurred in all mice receiving both estrogen and the same preparation of prolactin.

The rudimentary glands were not stimulated in the 12 operated animals receiving either of the 2 preparations of highly purified prolactin (SP and AW) at any of the 3 different dose levels. The failure of mammary growth to occur in hypophysectomized male mice given purified lactogenic hormone is in agreement with the observed lack of growth of the mammary glands in hypophysectomized rats injected with large amounts of prolactin.[4] The 13 hypophysectomized mice receiving estrogen in addition to the purified lactogenic preparations showed mammary growth rated as ++ in 11 animals and + in the other 2. At the

TABLE 10

MAMMARY RESPONSES IN HYPOPHYSECTOMIZED MALE MICE RECEIVING VARIOUS HORMONES AND EXTRACTS ALONE AND TOGETHER WITH ESTROGEN

Number of Mice	Treatment*	Period treated	Number of mice showing mammary growth
		Days	
13	Saline pituitary extract	9–16	3
17	Saline pituitary extract + estradiol dipropionate	4–16	17
5	Prolactin^{-SC} (Schering—12.5 I.U./mg.)	11–12	3
5	Prolactin^{-SC} (Schering—12.5 I.U./mg.) + estradiol dipropionate	11–12	5
8	Prolactin^{-SP} (Schering—35 I.U./mg.)	11–12	0
12	Prolactin^{-SP} (Schering—35 I.U./mg.) + estradiol dipropionate	9	12
4†	Prolactin^{-AW} (35 I.U./mg.)	10	0
1	Prolactin^{-AW} (35 I.U./mg.) + estradiol dipropionate	10	1
5	Saline fetal extract (mouse)	9–12	0
2	Saline fetal extract (mouse) + estradiol dipropionate	9	0
2	Thyrotropic hormone	9	0
3	Thyrotropic hormone + estradiol dipropionate	9	0
2	Thyrotropic hormone + prolactin	9	0
2	Thyrotropic hormone + prolactin + estradiol dipropionate	9	2

* Estradiol dipropionate, 1 μg. every other day. Pituitary hormones given daily. Prolactin given at dose levels of 0.1, 0.3, and 1.0 mg. daily with uniform results. Thyrotropic hormone given in doses of 0.25 mg. daily. Saline pituitary extract given in doses of 0.25 mg. total solids daily. Saline fetal extract given in doses of 0.2 to 2.2 mg. total solids daily.

† This prolactin preparation has been demonstrated to be homogeneous. White, A., Bonsnes, R. W. & Long, C. N. H. Jour. Biol. Chem. 143: 447. 1942.

time they were removed the glands were growing rapidly as indicated by the enlarged end-buds containing numerous mitotic figures. The extent of mammary proliferation averaged somewhat less than that obtained in unoperated mice receiving estrogen, and was comparable to that induced in hypophysectomized mice receiving estrogen and progesterone.[61]

It has further been observed in these studies that a highly purified preparation of pituitary thyrotropic hormone, containing significant amounts of gonad-stimulating material, failed to produce mammary gland growth in hypophysectomized male mice receiving estrogen. The inability of these two pituitary hormones to stimulate mammary growth under the experimental conditions employed confirms the observations of Reece and Leonard[6] with hypophysectomized rats. Three of 13 hypophysectomized male mice receiving saline extracts of pituitary tissue showed mammary growth. All mice given estrogen and the saline extract showed mammary growth comparable to that in mice receiving estrogen and prolactin.

Further evidence that prolactin is the limiting factor in mammary development in these studies is seen in the experiments in which thyrotropic hormone and estrogen, either alone or in combination, did not induce mammary growth, whereas the addition of prolactin to these two other hormones produced mammary proliferation.

THE PRESENT STATUS OF THE MAMMOGEN HYPOTHESIS

In a discussion of the question of the existence of a specific anterior pituitary mammogen factor or factors regulating the development of the mammary gland, it is obviously necessary to consider the following questions:

(1) Can an adequate explanation of mammary development be made which is based on known hormonal factors without the inclusion of the mammogen hypothesis?

(2) In the event that the answer to the first question is an affirmative one, can the experimental data supporting the mammogen hypothesis be rationalized from the information available, disregarding the postulation of new hormones?

(3) In the event that the answer to the first question is affirmative, and to the second negative, is it justifiable to assume that there may be several different anterior pituitary hormones controlling the development of the mammary gland?

The experiments which have been described with prolactin demonstrate for the first time that highly purified lactogenic hormone admin-

istered with estrogen to hypophysectomized male mice will produce mammary growth in these animals. Inasmuch as the prolactin alone has no effect, and the injection of estrogen into hypophysectomized animals produces no marked mammary stimulation, it is evident that the action of both of these hormones together is required. The conclusion may be drawn that prolactin may either sensitize the mammary tissue of hypophysectomized mice to estrogen or improve the general condition of these animals so that mammary proliferation may occur. Whatever the correct interpretation of the results may be, the need for both the pituitary and the ovarian factors in these experiments seems evident. It should be kept in mind, however, that experimentally the pituitary factor may be replaced by either progesterone or desoxycorticosterone acetate, since either of the latter steroids will produce mammary growth in hypophysectomized male mice treated with estrogen.[61] Moreover, the nonessential role of the pituitary has been emphasized by the production of mammary development by direct application of estrogen to the skin. This is not to be construed, however, as minimizing the importance of the pituitary gland or its secretions in normal mammary gland physiology.

The preceding discussion pertains to evidence obtained in experimental animals. It is quite obvious that a number of endocrine glands, *i.e.*, the pituitary, the adrenals, and the gonads, and other factors may contribute to complete development of the mammary gland. It is also obvious that inasmuch as under normal circumstances the secretion of adrenal cortical steroidal substances is under the influence of the anterior pituitary, pituitary factors other than prolactin may influence mammary development. Yet another possibility has little experimental support, namely, that prolactin itself may influence adrenal cortical activity.

Although it is true that previous experiments with prolactin and estrogen have yielded negative results in mammary growth studies, the body of positive evidence previously obtained with crude and now with highly purified prolactin permits the conclusion that an adequate explanation of mammary development may be made on the basis of the effects of combinations of several known hormones, *e.g.*, prolactin and estrogen or progesterone and estrogen, on the mammary gland.

With the first proposed question now answered in the affirmative, we come to the second, and somewhat more difficult one, namely, the rationalization of the experimental data supporting the mammogen hypothesis solely on the basis of experimental conclusions which do not include the existence of the mammogen complex.

The evidence for the presence in anterior pituitary tissue of a lipid-

soluble factor which produces growth of the mammary gland must, to be accepted, preclude several other possible explanations of the data. One of these necessitates an examination of the methods of preparing mammogen to ascertain whether the extraction methods would remove any of the known anterior pituitary hormones, *e.g.*, the proteins and more specifically, prolactin. The use of organic solvents in which proteins are generally insoluble would appear to limit this possibility. It is true, however, that the extraction of fresh pituitary tissue with organic solvents is essentially an extraction with a mixture of water and organic solvent, and the extractability of the pituitary protein hormones with aqueous alcohol and aqueous acetone is very great indeed. The fact that such extracts are subsequently increased in organic solvent concentration to as much as 80 per cent, in terms of alcohol or acetone, and at a pH at which the anterior pituitary protein hormones exhibit minimum solubility, does not permit one to ignore the definite and significant solubility of certain of these proteins under these conditions. Concentration of supernatants, after removal of precipitated proteins, may therefore serve to concentrate small amounts of pituitary protein hormones in these solutions.

Notwithstanding the above intimation that there may be certain anterior pituitary proteins in mammogen preparations, one of which is now established as functioning in mammary development, the technique used by Turner and his colleagues for further concentration of mammogen would appear to eliminate an explanation of their data on the basis of the presence of pituitary protein hormones in mammogen preparations. Lewis and Turner[27] reported that oily residues, obtained by the evaporation of aqueous-organic or organic solvent extracts of pituitary tissue or of supernatants remaining after precipitation of pituitary proteins, could be further purified by means of ether extraction. Moreover, the mammogenic activity was in the ether-soluble fraction; the ether-insoluble residue gave negative results on assay for mammogen. In view of these latter observations, the presence of protein material in the active mammogen preparations seems unlikely, although the solubility of proteins in lipid solvents, in the presence of lipoidal material, is not unknown.

Let us now turn to an examination of the possibility that known lipid-soluble hormones may be present in the pituitary gland and may account for the effectiveness of mammogen preparations in mammary development. The mammary growth-promoting action of estrogen, progesterone and adrenal cortical hormones makes valid this inquiry. Although steroid hormones have not been isolated in pure form from pit-

uitary tissue, there is ample physiological evidence for their presence in significant amounts. Brouha and Simonnet,[67] in 1927, reported that the subcutaneous administration of 20 mg. of lipid-soluble extract of cattle pituitaries into immature rats induced vaginal opening and cytological changes of the vagina characteristic of the estrus cycle and development of the uterus. In adult female castrate rats 50 to 100 mg. of material resulted in a complete estrus cycle. Callow and Parkes[68] obtained similar results in 1936 when they observed that an acetone extract of the hypophyses of cattle proved to be highly estrogenic. Moreover, Lewis and Turner[52] have investigated the possible estrogenic potency of fresh anterior pituitary tissue and extracts, using vaginal smear reactions in ovariectomized mature mice and increase of uterine weight in immature mice as criteria of estrogenic potency. Approximately 17 per cent of the test animals showed estrus vaginal smear reactions and the presence of a small amount of estrogen was indicated by the uterine weight test. It should be pointed out, however, that the majority of the positive responses were obtained with a single preparation, whereas each of 4 other extracts gave negative vaginal smears.

Qualitative examination of anterior pituitary tissue and extracts having mammary growth activity for the presence of progesterone has recently been reported by Turner and his colleagues.[69] This seemed highly desirable in view of the observation of Gardner and Hill[70] that progesterone alone stimulates the growth of the duct system of the mammary gland. But the Missouri group found that amounts of fresh pregnant cattle pituitary which will stimulate growth of the lobule-alveolar system in castrate female mice did not contain sufficient progesterone to give a positive response by the sensitive McGinty technic. Lipid extracts of the anterior pituitary which stimulated duct growth in the male mouse were also found to be negative for progesterone. These observations were taken to indicate that the mammogenic effects of the anterior pituitary are not due to progesterone. It should not be overlooked, however, that the presence of other sex hormones in these extracts may inhibit the progesterone response.

In view of the demonstrated presence of estrogenic hormone in the pituitary, and its occurrence in the lipid concentrates employed by Turner and his collaborators, and inasmuch as normal male and castrate female mice have been the assay animals for demonstration of mammogen activity, it is evidently possible that the functioning of the pituitary in

[67] **Brouha, L., & Simonnet, H.** Comp. rend. Soc. Biol. **96**: 1275. 1927.
[68] **Callow, R. K., & Parkes, A. S.** Jour. Physiol. **87**: 288.* 1936.
[69] **Trentin, J. J., Mixner, J. P., Lewis, A. A., & Turner, C. W.** Proc. Soc. Exp. Biol. Med. **46**: 440. 1941.
[70] **Gardner, W. U., & Hill, R. T.** Proc. Soc. Exp. Biol. Med. **34**: 718. 1936.

these mice supplied one of the pituitary hormones, *e.g.*, prolactin, whose synergistic effects with estrogen on mammary gland development has now been established. However, in a recent publication Lewis, Gomez, and Turner[71] have compared mammary gland development with mammogen in the castrate and in the hypophysectomized rat. It was demonstrated that lipid extracts of anterior pituitary glands from pregnant cattle produce mammary duct growth in both castrate and in hypophysectomized rats. The castrate animals were males of varying ages; the hypophysectomized animals were young male and female rats. There was no significant change in the weights of the thyroids, ovaries and uteri of the treated hypophysectomized female rats as compared with similar uninjected controls. Adrenal gland weights of treated rats appeared to be somewhat smaller than those of the control group of animals.

The demonstration of the effectiveness of lipid pituitary extracts in producing mammary growth in hypophysectomized rats appears to rule out a pituitary influence in these animals, particularly in view of the already discussed improbability of the presence of protein pituitary hormones in these extracts. But the demonstrated capacity of certain steroid hormones to produce mammary growth must be kept in mind. TABLE 11 is compiled from data in the publication of Turner and his colleagues. It will be observed that negative results on mammary duct growth were obtained in castrate male rats given Mammogen I in a total dose of 1.8 m.u. (mouse units) in 6 injections. But when larger total doses were given for longer period of time (0.18 to 9.0 m.u. of Mammogen I daily for 28 to 30 days), positive duct-end buds and considerable duct development was seen at the higher dosages. The percentage response was not high until several mouse units a day were given for 16 to 30 days. The lipid extracts employed are reported to have an activity of 1 Mammogen I mouse unit in 0.1 to 1.0 mg. of material. In other words, an animal given 0.5 mouse units daily may have received from 0.05 to 0.5 mg. daily, depending on the activity of the particular preparation employed. Over a period of average length of 23 days, the total amount of material injected would have varied from 1.2 to 12.0 mg. In the experiments with hypophysectomized rats, 4 to 16 m.u. were given daily for 7 to 10 days. This is an average total dose of approximately 8 to 80 mg. per animal, depending on the activity of the particular mammogen preparation employed. Similar relatively large dosages have been used in previous experiments from Turner's laboratory demonstrating a lipid-soluble mammogen. If 0.1 per cent of the lipid-soluble

[71] **Lewis, A. A., Gomez, E. T., & Turner, C. W.** Endocrinology **30**: 37. 1942.

TABLE 11

EFFECT OF MAMMOGEN I EXTRACT ON THE MAMMARY GLANDS OF CASTRATE AND OF HYPOPHYSECTOMIZED RATS[71]

Type of rat	Number of animals	Dose per day (mouse units)	Number of injections	Total dose (mouse units)	Mammary change
Male, castrate	3	untreated	0		Regression or no change
" "	3	1.8 cc. olive oil	6		Regressed
" "	8	0.003–0.03	6	0.018–0.18	No duct development
" "	9	0.07–0.3	6	0.42–1.8	Appeared active; no end buds
" "	18	0.006–0.3	30	0.18–9.0	5 positive; end buds
" "	9	2.0–8.0	28	46–224	3 positive; end buds
Male, hypophysectomized	3	0			Ducts atrophic
" "	4	4–16	7–10	28–160	Positive
Female, hypophysectomized	11	0			Ducts atrophic
" "	12	4–16	10	40–160	Positive

material consisted of steroid hormone substances, the variable positive mammary growth observed might be expected.

The possible general occurrence of the steroid hormones in small amounts in various tissues, and their effect in very small dosages on the mammary gland must be kept in mind when evaluating results which are interpreted as indicating the existence of new specific factors concerned with mammary gland development. Furthermore, Nelson[72] has recently presented additional physiological evidence demonstrating that sex hormones are produced in the adrenal cortex. These experiments supplement the isolation experiments of Reichstein, of Wintersteiner, and of Pfiffner, and studies by others of the urines of patients with pathological changes of the adrenals. The latter endocrine organs, on the basis of already available evidence, may assume an important role in mammary gland physiology. It may also be pointed out that Lewis and Turner[2] were able to cause mammary hyperplasia in male mice with lipid-soluble extracts of guinea pig livers, although it is true that 80 to 90 mg. of material were required to produce a positive response in 30 per cent of the animals used.

In summing up the present available evidence in an effort to obtain an answer to the second proposed question, namely, can the data supporting the mammogen hypothesis be rationalized from this evidence (disregarding the postulation of new hormones) the following points may be made:

(1) The wide distribution of varying quantities of steroid hormones in the body tissues, and the synergistic and broad physiological actions of certain of these hormones, make it necessary to exclude completely their possible presence in any crude extracts alleged to contain new lipid-soluble hormonal factors. This is particularly true when:

(a) Small amounts of certain known steroid hormones are demonstrable in lipid extracts of various tissues.

(b) Relatively large quantities of lipid-soluble fractions are required to produce a given mammary gland response in studies designed to support claims for new factors.

(2) It has been established that certain endocrine organs, *e.g.*, the adrenal cortex, produce several types of physiologically active steroids (estrogens), some of which may have a main site of production elsewhere in the body. Under these circumstances, removal of the principal locus of formation of a particular hormone does not completely eliminate this hormone from the physiological picture.

(3) The data which have been presented in support of the mammogen

[72] **Nelson, W. O.** Anat. Rec. **81**: 97. Suppl. 1941.

hypothesis may find explanation in the established roles of certain hormones, *e.g.*, prolactin, estrogens, progesterone, and desoxycorticosterone, in mammary gland growth and development, and the possible but as yet unproven roles of other hormones, *e.g.*, corticosterone, adrenotropic hormone of the anterior pituitary, in these processes.

(4) In view of the foregoing, it is concluded that the existing evidence for the secretion of a lipid-soluble mammogen complex by the anterior pituitary gland must be supplemented by: (a) purification of and more intimate chemical information regarding the nature of the lipid-soluble extracts of this tissue, and (b) a more highly refined experimental elimination of possible influences of known hormonal factors in physiological experiments designed to obtain evidence for the mammogen hypothesis.

THE GROWTH AND METABOLIC HORMONES OF THE ANTERIOR PITUITARY*

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INTRODUCTION

The discussion of the anterior pituitary factors that influence the growth and metabolism of animals is one of unusual complexity. In the first place, it is obviously impossible to distinguish between effects described as "growth promotion" and those that are termed effects on metabolism, since in the last analysis growth in the true sense is dependent on chemical reactions involving not only the synthesis of cellular components but also those by which energy is provided for such synthesis. Furthermore, the term growth has only a general meaning and unless more rigidly defined contributes but little to our understanding of the role played by the endocrine glands in its occurrence and continuance.

When true growth occurs the cells of an individual organ or those of a larger part of the animal increase not only in size but also in number. This means that not only is the rate of accumulation of the characteristic cellular constituents (mainly protein, salts, and water) increased but also their absolute quantities in the organism. This continues until the animal has reached adulthood, at which time the quantity of certain constituents of the body, notably protein and inorganic salts, remains constant although the animal may continue to increase in size and weight by the addition of fat. It is important to remember that, although the quantity of protein remains constant, this constituent of protoplasm is now known to be undergoing constant breakdown and resynthesis. This knowledge has recently been gained by the brilliant work of Schoenheimer and his colleagues, who made use of the heavy isotope of nitrogen, and this information is of the utmost importance not only to our understanding of protein metabolism but also because it indicates that those processes by which protein is formed during the period of rapid growth

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do not cease at adulthood but continue with the addition of some form of regulation so that the total quantity of protein in the organism is maintained within fairly narrow limits. This regulation might be accomplished by the recession of some agent that normally stimulates the anabolism of protein or by the increased activity of some factor that increases tissue catabolism, the result in either case being the establishment of a steady state.

It is now well known that the composition of the organism is not similar at different periods of growth. Moulton and his colleagues [2] have made detailed studies of the composition of the gain in weight of animals at different periods, and their findings are summarized. Intra-uterine growth is characterized by a relatively high proportion of water (90 per cent), a relatively low content of protein (5–9 per cent) and ash (1–2 per cent), and the deposition of fat is quite small (1–2 per cent). After birth the proportion of water in the material comprising the gain in weight decreases quite rapidly while that of protein increases to some 15 to 20 per cent. As the age of the animals advances, increasing quantities of fat are deposited and since fat is laid down with comparatively small quantities of water, the water content of the animal declines still more. When true growth ceases protein and ash are no longer accumulated but some animals, including man, may continue to increase in size and weight by further addition of fat.

Since the continued addition of fat dilutes as it were the true composition of growth, Moulton[1] has calculated for several species both the composition of the whole animals and the composition of the gain in weight on a fat-free basis. This method leads to some interesting conclusions for it is found that the proportions of protein and ash increase steadily during intra-uterine life and during the early period of extra-uterine existence. Following this, a period is reached in which the proportions of protein, ash and water remain remarkably constant, although as pointed out above very large quantities of fat may be accumulated. It is this period that Moulton has termed the "age of chemical maturity."

The interest in the steady state of the composition of the body of adult animals has been increased by the fact that the turnover of many of the elements, particularly the proteins, is continuous throughout life and consequently it may be assumed that rather exact regulatory mechanisms operate during the adult period. Among these are undoubtedly certain endocrine glands and in particular the anterior pituitary.

If it be allowed that the chemical characteristics of true growth are the

[1] **Moulton, C. R.** Jour. Biol. Chem. **57**: 79. 1923.
[2] **Armsby, H. P., & Moulton, C. R.** "The Animal as a Converter of Matter and Energy." Am. Chem. Soc. Monograph Series, No. 23. The Chemical Catalog Co. New York. 1925.

accumulation of inorganic elements, proteins and water,[3] we are enabled by studies of the water, ash and protein metabolism of the organism to analyze the influence of any agent that either enhances or inhibits growth. It may even at this point be stated that anterior pituitary extracts do not qualitatively alter the composition of true growth but merely increase its proportion in the total weight gain.

THE ANTERIOR PITUITARY AND GROWTH

It will be agreed that the anterior pituitary is only one of many agents that determine the ultimate size and form of the whole animal and its component organs. Furthermore, there is good reason to believe that the presence of a pituitary factor is not always essential to growth. This is certainly true during the early stages of embryonic existence[4] and though the importance of the pituitary factor increases steadily in the later stages, it is now known that hypophysectomy of animals soon after birth does not lead to an immediate cessation of growth. This is particularly true in the rat where the effects of this operation have been most extensively studied. But if hypophysectomy is performed when the rats have reached a weight of 80-100 gm., growth ceases immediately even though the animal is supplied with a diet that is entirely adequate for normal growth of intact animals.

These facts are well known but the picture is not so simple as it appears at first sight. In the first place, removal of the hypophysis also removes certain other hormones whose presence is essential for the normal functions of such endocrine glands as the thyroid, adrenal cortex and gonads. These not only undergo anatomical regression but also it can be demonstrated that their function is very much reduced although not entirely obliterated. As is well known, normal growth is not possible in the absence of sufficient quantities of either the thyroid or adrenal cortical hormones and the question might well be asked, whether the cessation of growth after hypophysectomy is not due partly to the multiple glandular deficiency that follows this procedure.

In addition, hypophysectomized animals have poor appetites and consume much less food than normal animals. Whether this is a cause of growth retardation or merely a consequence of the profound changes in metabolism has not been entirely answered, but in all probability it

[3] It is of course apparent that many other organic substances are accumulated as the organism grows, but the proteins by virtue of the unique position they occupy as constituents of protoplasm are perhaps the most important for studies of this kind. It should also be noted that the entire exclusion of lipids from the composition of true growth is also not justified since many of them are essential constituents of cells. Nevertheless, the available data suggest that the essential lipid components form only a small proportion of the gain of rapidly growing animals and that their exclusion does not greatly alter conclusions regarding the composition of the material laid down in these periods.

[4] **Fugo, N. W.** Jour. Exp. Zool. **85**: 271. 1940.

should be regarded as an adjustment of the organism to its new metabolic environment. It should also be recalled that removal of the hypophysis does not prevent regeneration of certain organs such as the liver. This has been clearly shown by Franseen, Brues and Richards[5] and Higgins and Ingle.[6] Both studies agree, however, that there is some retardation of the rate of regeneration which may in part be due to the smaller food consumption of hypophysectomized rats.

The most convincing evidence of the essential character of an anterior pituitary factor for normal growth is furnished by the effect of injections of extracts of this gland into hypophysectomized and normal animals. In the former case, growth is immediately resumed; in the latter, accelerated growth rates occur in young animals and those that had attained a stationary state begin to grow again. But even in these animals the part played by stimulation of other endocrine glands in the total response to the extract still needs to be taken into consideration.

It is necessary to take up in some detail the nature of the response to growth-promoting extracts in order that not only the essential action of the growth hormone may be understood but also the relation of this to the other metabolic effects of the anterior pituitary.

THE MODE OF ACTION OF THE GROWTH HORMONE OF THE ANTERIOR PITUITARY

General Effects of Growth-Promoting Extracts

Although the first association of the anterior pituitary with growth promotion was noted in individuals suffering from pituitary tumors, the first experimental demonstration that the growth rate of mammals could be accelerated by the injection of anterior pituitary extracts was carried out by Evans and Long,[7] who showed in 1921 that true gigantism could be produced in rats by this means. Later it was shown, particularly by Teel and his collaborators,[8] as well as by Evans, *et al.*[9] that injections of similar extracts into dogs produced not only an increased growth rate but also a condition strikingly similar to acromegaly in man.

Since most work has been and now is being carried out on rats, it may be well to outline the course of events in this species following injection with anterior pituitary extract (A.P.E.). Female rats that have reached a nearly constant weight (plateaued) are usually used. Males also respond but are less sensitive than the females. Growth is accelerated at once and in the first experiments reported the treated animals ultimately

[5] **Franseen, C. C., Brues, A. M., & Richards, R. L.** Endocrinology **23**: 292. 1938.
[6] **Higgins, G. M., & Ingle, D. J.** Anat. Rec. **73**: 95. 1939.
[7] **Evans, H. M., & Long, J. A.** Anat. Rec. **21**: 62. 1921.
[8] **Putnam, T. J., Benedict, E. B., & Teel, H. M.** Arch. Surg. **18**: 1708. 1929.
[9] **Evans, H. M., et al.** Mem. Univ. California, **11**. 1923.

reached a weight almost double that of their litter sisters. This growth was symmetrical, that is, the increase in length of the animal was proportional to the increased weight. While not much data have been collected on this point, such as are available are presented in TABLE 1 and are contrasted with the unsymmetrical "growth" found in rats after hypothalamic puncture. It will be observed that the A.P.E.-treated animals have ratios of body weight to body length that are somewhat greater than those found in untreated animals of the same length, a finding which indicates that the animals may have been slightly obese. As will be seen, however, there is ample evidence that true growth was induced by this treatment.

TABLE 1

WEIGHT-LENGTH RELATIONSHIPS OF:
(a) RATS TREATED WITH GROWTH HORMONE AND
(b) OBESE RATS FOLLOWING HYPOTHALAMIC PUNCTURE[10]

	Weight	Length	$\frac{\text{B.W.}}{\text{B.L.}}$ ratio
	grams	*norms*	
Normal males	372	236	1.58
" "	448	250	1.79
Males injected with A.P.E.	485	255	1.90
Normal females	220	204	1.08
" "	350	230	1.52
Females injected with A.P.E.	397	232	1.71
Normal females	300	216	1.39
Obese female litter mates	403	207	1.95

[10] Data compiled from: **Donaldson, H. H.** "The Rat." Mem. Wistar Inst., Philadelphia. No. 6, 1924; **Evans, H. M., & Simpson, M. E.** Am. Jour. Physiol. **98**: 511. 1931; **Tepperman, J., Brobeck, J. R., & Long, C. N. H.** Unpublished data.

At this point it is necessary to call attention to an anomalous situation. In the first experiments reported by Evans and Long the rats were injected daily for as long as 8 to 13 months with a crude alkaline extract and although growth was not as rapid in the late period as at first, nevertheless it was continuous throughout the period of injections. Later attempts to repeat this experiment, even in the same laboratory, showed that, after an initial period of brisk growth, the animals became refractory to the extract and even lost some of the weight they had gained. The same result was also obtained in hypophysectomized animals but even more discouraging was the fact that partial purification of the extract did not correct this decreased responsiveness. The development of a refractory state to anterior pituitary extracts is not limited to their growth-promoting properties and has been shown to be true for the stim-

ulation of thyroid and gonads that follows their use. This whole subject has been developed by Collip and others in an interesting manner and Collip has coined the term, "anti-hormones," to describe the substances that appear in the serum of such animals. The reader is referred to a recent review by Thompson[11] for a full description of present views on this subject.

In hypophysectomized animals there may be additional reasons for the ultimate failure to respond to growth-promoting extracts. Evans and his colleagues[12] have shown that many hypophysectomized animals will again become responsive if crude extracts are substituted for the more purified preparations or if they receive glucose injections. It should also be remembered that hypophysectomized animals have atrophic adrenals and thyroids and that, unless these keep pace with the increased size and metabolic demand, growth may ultimately cease, since it is hard to conceive of any animal growing to adult size when deprived of all but minimal amounts of the hormones of these glands. Consequently, while purification and separation of the growth-promoting agent from the adrenotropic and thyrotropic agents can be achieved, it may be anticipated that these extracts over a long period will prove to be inadequate to support continuous growth in hypophysectomized and possibly in normal animals by reason of the fact that the adrenals and thyroid do not receive a stimulation proportional to that induced by the growth principle in the somatic tissues. This, however, does not exclude the fact that such a preparation may induce an immediate but temporary increase in growth.

In a recent paper Marx and coworkers[13] have studied the growth response of hypophysectomized rats to the injection of a growth hormone preparation that contained only small quantities of the other anterior pituitary hormones. The treated animals were pair-fed with a control group. While their body weight increased 25 per cent it was also found that this was attended by an increase in weight of different degrees in various organs, including the ovaries, adrenals and thyroids. The increase in the endocrine glands is particularly interesting inasmuch as the preparation injected apparently contained only minute amounts of tropic hormones. This raises the question as to whether a tropic hormone is not more concerned with the formation and liberation of specific hormones than to the actual size of the endocrine glands that produce them.

As mentioned above, the role played by the quantity and quality of the

[11] Thompson, K. W. Physiol. Rev. **21**: 588. 1941.
[12] Evans, H. M., Pencharz, R. I., & Simpson, M. E. Endocrinology **19**: 509. 1935.
[13] Marx, W., Simpson, M. E., Reinhardt, W. O., & Evans, H. M. Am. Jour. Physiol. **135**: 614. 1942.

diet in determining the response to either hypophysectomy or the injection of A.P.E. has not yet been entirely worked out. Bryan and Gaiser[14] have pointed out that the degree of response of rats is conditioned in part by the diet fed and even without growth hormone rats may, by improvements in the diet, be induced to grow to a size similar to that achieved by A.P.E. on ordinary diets.[15] Lee and Schaffer[16] have clearly shown that on a pair-fed regime, however, rats injected with A.P.E. grow more than do untreated animals. This has also been shown to be true for pair-fed hypophysectomized rats.[13]

Effects on Special Organs and Tissues

The fact that hyperactivity of the pituitary gland is associated with marked changes in certain organs and tissues of the body has been known for some time. The bony deformities, splanchnomegaly and enlarged tongue of individuals suffering from acromegaly are well known stigmata of this disease. Putnam, Benedict and Teel[8] found in a dog in which a condition resembling acromegaly was produced by chronic injection of A.P.E., that the liver was enlarged more than any other organ except the gonads and thyroid, for which organs the extract contained specific principles. The liver showed evidence of a central necrosis. Downs[17] has also noted the disproportionate increase in the liver of treated mice. The cell nuclei were greatly increased with a slight increase in cell size. There was also a marked central necrosis. In dogs there was also a marked increase in the size of the liver and central necrosis.

In experiments conducted over a short period (1–16 days) in pair-fed rats Lee and Freeman[18] have found that, although the average liver weight was significantly greater in treated animals, the number of cells per gram of tissue remained unchanged and they conclude that the liver exhibits a true hyperplasia under the influence of the extracts used and that all elements of the organ participate in it. The total quantity of nitrogen in the liver per 100 gm. of rat was 117 mg. in the controls and 129 mg. in the treated animals, indicating that in proportion to their body weight the latter had accumulated more nitrogen (protein) in their livers.

The bony changes accompanying hyperpituitarism in man have always excited interest. As is well known these are of two types: (1) a general increase in the size of the skeleton that accompanies gigantism and which occurs only when the epiphyses are open; and (2) the bony deformities

[14] **Bryan, A. H., & Gaiser, D. W.** Am. Jour. Physiol **99**: 379. 1932.
[15] **Anderson, W. E., & Smith, A. H.** Am. Jour. Physiol. **100**: 511, 1932.
[16] **Lee, M. O., & Schaffer, N. K.** Jour. Nutrit. **7**: 337. 1934.
[17] **Downs, W. G., Jr.** Jour. Dent. Res. **10**: 601. 1930.
[18] **Lee, M. O., & Freeman, W.** Endocrinology **26**: 493. 1940.

associated with acromegaly which develop when hyperpituitarism occurs during adult life.

Putnam, Benedict and Teel[8] used bulldogs for their chronic experiments and found that the treated animals exhibited marked bony deformities. The bones were thickened and heavy and local changes such as are seen in human acromegaly were present. Evans and his colleagues[9] suggested that the choice of the bulldog for such injections may have influenced the results, as this animal may be regarded as already possessing acromegalic traits. These investigators injected a more normal type of dog (shepherd) and reported that in this breed the prolonged injection of A.P.E. did not lead to any appreciable changes in the long bones although the skull bones were enlarged. In the dachshund marked general growth was produced but the achondroplastic form of the short extremities was retained. The long bones were not particularly affected but those of the skull were thickened and enlarged. In both of these breeds the most pronounced changes occurred in the soft tissues and it is of exceeding interest that diabetes mellitus was observed to appear in two dogs after some months of treatment.

Mortimer[19] has made extensive X-ray studies not only on the changes produced by A.P.E in the skulls of normal rats but also those that follow hypophysectomy in this species. The skulls of the treated rats had less density and were more highly developed than the controls, but as Mortimer points out, the crude extracts used contain factors that stimulate the thyroid and possibly the parathyroids so that care must be used in the interpretation of the results. The changes in the skull after hypophysectomy are, however, quite characteristic. There is a marked decrease in vascularity, and the processes of bone resorption and deposition are unbalanced. All growth does not cease but the skull grows more in its transverse and vertical direction than it does in its anteroposterior diameters so that the head retains its infantile proportions and appearance. The eruption of the teeth is retarded, their outline deformed and the pulp cavities obliterated. Similar tooth changes have been described by Schour and Van Dyke,[20] and Downs[5] has reported that the injection of A.P.E. into dogs hastened the eruption of the teeth. In mice similar treatment led to a noticeable increase in the size and density of the incisor teeth and maxillae.

Histological studies on the changes in the epiphyses and related structures following hypophysectomy or treatment with A.P.E. have been

[19] **Mortimer, H.** Radiology **28**: 5. 1937.
[20] **Schour, I., & Van Dyke, H. B.** Am. Jour. Anat. **50**: 397. 1932.

made by several investigators. The Silberbergs[21] in particular have made extensive studies of both guinea pigs and mice. They found that extracts of beef anterior pituitary increase proliferation and may induce degenerative changes in the epiphyseal and articular cartilage and that they stimulate the deposition of bone. These effects were not prevented by thyroidectomy or ovariectomy. Freud, Levie and Kroon[22] have reported that after hypophysectomy in rats growth ceases in the tail vertebrae and that differences in the tail length can readily be detected by skiagrams as early as 7 days after operation. They also made the statement, which is somewhat surprising in view of the experience of other investigators, that the epiphyses close soon after hypophysectomy and that once completed, growth can no longer be induced by growth-promoting extracts, but if a growth-promoting extract is given immediately after hypophysectomy epiphyseal closure is prevented and tail growth continues. Not only have these investigators proposed that alterations in tail length and vertebral development be used as a method for the assay of the growth hormone but state that the growth defect after hypophysectomy is definitely localized in the epiphyseal cartilage. Consequently, they suggested that the growth hormone was a "chrondotropic" hormone, a view which was subsequently modified[23] to admit that the hormone has other sites of action. Levie and Uyldert[24] reported that removal of the adrenals does not prevent the usual effects of purified growth hormone on tail growth.

Ross and McLean[25] found that the administration of a growth preparation to plateaued rats induces histological evidences of active growth in the quiescent epiphyseal cartilage and adjacent spongiosa. They stated that this resumption of growth in the cartilage is often a better indicator of the activity of the preparation than an increase in body weight. These authors also conceded that the effect of the hormone upon the growth apparatus in the bone does not exclude the presence of specific effects in other tissues. Ray, Evans and Becks[26] have recently presented a detailed study of the alterations produced in the epiphyseal disc of rats by hypophysectomy and A.P.E.

The question of a specific pituitary factor influencing bone growth has been given a new turn by the recent observations of Ingalls and Hayes[27] that adrenalectomy is also followed by a failure of endochondrial bone

21 **Silberberg, M., & Silberberg, R.** Am. Jour. Path. **15**: 547. 1939; **16**: 491, 505. 1940; **17**: 189. 1940. Anat. Rec. **78**: 549. 1940. Endocrinology **29**: 475. 1941.
22 **Freud, J., Levie, L. H. & Kroon, D. B.** Jour. Endocrinology **1**: 56. 1939.
23 **Freud, J., & Dingemanse, E.** Acta brev. Neerland. **10**: 102. 1940.
24 **Levie, L. H., & Uyldert, I. E.** Acta brev. Neerland. **9**: 121. 1939.
25 **Ross, E. S., & McLean, F. C.** Endocrinology **27**: 329. 1940.
26 **Ray, R. D., Evans, H. M., & Becks, H.** Am. Jour. Path. **17**: 509. 1941.
27 **Ingalls, T. H., & Hayes, D. R.** Endocrinology **29**: 720. 1941.

formation similar to that following hypophysectomy. These authors suggested that the atrophy of the adrenal cortex that follows hypophysectomy may be a major factor in the interruption of growth. Levie and Uyldert found, however, that adrenalectomy did not interfere with the stimulating properties of growth hormone on tail length.

It is evident, as in many other instances of the effect of the anterior pituitary on metabolism, that the changes produced in bone growth by an excess or deficiency of its hormones are exceedingly difficult to interpret, partly by reason of the number of hormones involved that may alter the processes in bones but also by reason of the fact that the long-continued injection of crude extracts results in the development of refractory states which in some instances may actually produce a hypofunction of certain endocrine glands.

Metabolic Changes Induced by Growth-Promoting Extracts

The injection of crude anterior lobe extracts into animals is followed by marked and widespread alterations in metabolism. Since such extracts contain principles other than those associated with growth promotion and since no purified growth preparation has so far been extensively tested for its effects on metabolism it is extremely difficult to interpret the large number of observations that have been reported. An attempt will be made in a later section of this paper but for the moment particular consideration will be given to the alterations in protein metabolism. The reasons for this have been outlined above and it will be sufficient to repeat that true growth is always associated with the accumulation of protein in the body and, since this can be accomplished only by an absolute or relative change in protein synthesis, the indications seem clear that either directly or indirectly the growth hormone influences these processes. The evidence for such a participation of the growth hormone is quite conclusive and indeed is strongly suggestive that the true role of the anterior pituitary in the growth process is its influence on protein metabolism. The experimental facts are as follows:

(1) The injection of anterior pituitary extract into dogs is followed by a fall in blood N.P.N., urea and amino acids of some 20–30 per cent. This occurs within a few hours of injection and may persist for some time.[28] Harrison and Long[29] have observed a fall in the blood N.P.N. of fasted rats after A.P.E. injections.

(2) Gaebler[30] has found that not only is the blood N.P.N. decreased after a single injection of A.P.E. but there is an even more striking re-

[28] **Teel, H. M., & Watkins, O.** Am. Jour. Physiol. **89**: 662. 1929.
[29] **Harrison, H. C., & Long, C. N. H.** Endocrinology **26**: 971. 1940.
[30] **Gaebler, O. H.** Jour. Exp. Med. **57**: 849. 1933.

duction in the urine nitrogen excretion. This occurs even in phloridzinized dogs[31] and fasted rats.[29] In Gaebler's experiments the injections also lowered the respiratory quotient and raised the basal metabolic rate. There was marked diuresis and following the injection the greater part of the retained nitrogen was excreted within the next few days.

(3) Schaffer and Lee[32] stated that the injection of A.P.E. into normal rats causes a slight fall in the urea and amino acid content of the carcass and a much greater fall in that of the liver, the urea content being reduced to less than half the initial amount. The total N.P.N. was always reduced. Prolonged treatment with A.P.E. kept the amino acid and urea content of the liver at low levels.

(4) The changes in the composition of animals whose growth has been accelerated by A.P.E. have been studied by several investigators. The first of these studies appears to be that of Downs[17] who injected mice daily for 105 days with an alkaline extract prepared by the method of Evans and Simpson. Analysis of his data indicates that the composition of the gain of the treated animals contained more water, protein and ash, and less fat than did the control animals. Wadehn[33] performed similar experiments on mice. He concluded that the excess weight gained by the treated animals could not be attributed solely to fat or water retention since the animals contained, if anything, less fat than the controls. Bierring and Nielsen[34] used rats injected with A.P.E. daily for 7 months. Analysis of the gains in weight of the treated and control groups showed that the former deposited more water and less dry matter than the latter.

In all the above experiments the animals were fed *ad libitum* and as it is now known that those treated with A.P.E. consumed greater quantities of food the suggestion of greater water and less fat retention in the treated animals assumes some significance. The best and most quantitative studies of this character are those of Lee and Schaffer[16] on rats. The control and A.P.E.-treated animals were pair-fed for a period of 77 days and the composition of the gain in weight carefully determined. Their results which are presented graphically in FIGURE 1 are compared with the composition of the gain in weight of rats becoming obese after hypothalamic puncture.[35] They show quite clearly that the injection of A.P.E. caused not only a greater total gain in weight, but what is more important is that this gain was composed largely of water and protein (83 per cent) while fat constituted only some 13 per cent. In the control

31 **Gaebler, O. H., & Zimmerman, W. J.** Am. Jour. Physiol. **128**: 111. 1939.
32 **Schaffer, N. K., & Lee, M. O.** Jour. Biol. Chem. **108**: 355. 1935.
33 **Wadehn, F.** Biochem. Ztschr. **255**: 188. 1932.
34 **Bierring, E., & Nielsen, E.** Biochem. Jour. **26**: 1015. 1932.
35 **Hetherington, A. W., & Weil, A.** Endocrinology **26**: 723, 1940.

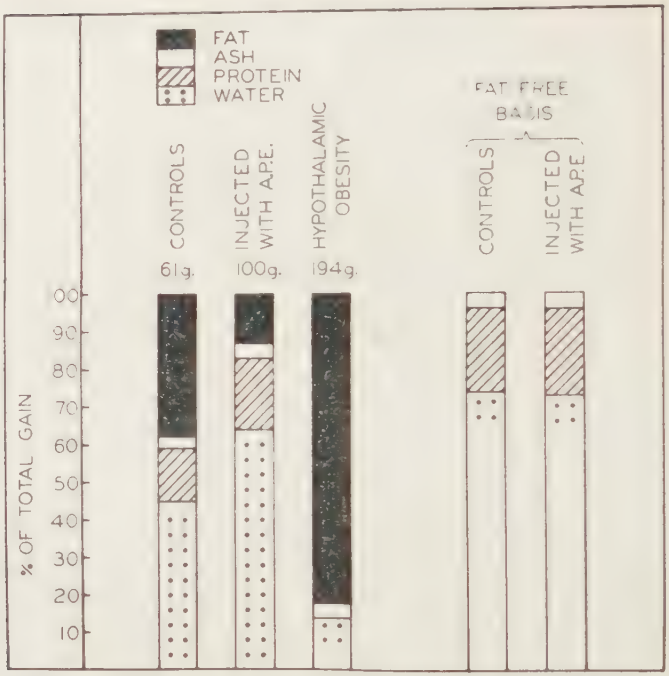

FIGURE 1. Composition of the gain in weight of rats after anterior pituitary treatment and hypothalamic puncture. (Data of Lee and Schaffer, Hetherington and Weil.

group water and protein made up 58 per cent of the gain while fat formed 39 per cent. In other words, under the influence of the extract the composition of the gain had reverted to that found in rapidly growing animals of a much younger age. It is also of interest to note that the extrauterine type of growth was retained and the embryonic type did not reappear, since the proportions of water, protein and ash in the gain in weight when expressed on a fat-free basis are unchanged by the treatment. This is also illustrated by a calculation of the water-nitrogen ratios as shown in TABLE 2. This ratio has a high value ranging from 60 to more than 100 in pig embryos, but after birth it falls to between 10 and 30, declining as the age of the animal increases and of course vanishing when nitrogen ceases to be retained.

The smaller increment of fat in the treated animals indicates, since the caloric intake was the same in both groups, that the treated animals had consumed a larger proportion of this substance to support their energy requirements. This stimulation of fat catabolism has been noted by all investigators to follow the injection of A.P.E. and its significance

TABLE 2
WATER-NITROGEN RATIOS OF THE GAIN IN WEIGHT AT DIFFERENT PERIODS OF GROWTH AND UNDER THE INFLUENCE OF THE GROWTH HORMONE

	H_2O	N_2	Ratio
Pig embryos			
10–15 mm. length	89.1	1.30	68.5
15–30 " "	90.6	1.14	80.1
30–50 " "	92.1	0.81	113.5
50–110 " "	90.9	0.93	97.6
110–160 " "	92.1	0.87	106.0
160–240 " "	87.3	1.40	62.2
Pigs after birth			
0–11 days	80.5	2.89	27.6
21–42 "	74.9	2.56	29.2
42–96 "	65.8	2.44	27.0
Mature pigs	22.0	1.03	21.5
Rats			
0–14 days	69.0	2.39	28.9
14–21 "	67.0	2.27	29.5
21–42 "	63.0	(1.48)	(42.6)
42–110 "	53.0	3.14	16.9
110–230 "	35.8	2.59	13.8
Rats			
(Lee and Schaffer)	45.0	2.01	22.4
A.P.E.-treated controls	63.3	3.15	20.1

in relation to other metabolic activities of the anterior pituitary will be considered later.

(5) Gregory and Goss[36] found that the injection of A.P.E. into rats or rabbits causes a 50 per cent decrease in the glutathione content of the liver in 12 hours. This observation is worthy of comment although the role of this substance in metabolism is not clearly understood at present.

To summarize, it is evident that the injection of anterior pituitary extracts rich in growth-promoting activity is accompanied by marked alterations in the metabolism of proteins and related compounds. These changes indicate that under the influence of these extracts either there is an increased synthesis of protein or the normal rate of protein catabolism is reduced. It is particularly noteworthy that these effects are not limited to animals receiving food containing protein but may also be observed during fasting or in animals that have been phloridzinized. Nitrogen retention under these last circumstances is not so marked as in well-fed animals but the fact that it is still present would indicate that the action of the hormone is not exactly expressed by the term "growth-promoting" but that it is in some manner intimately associated with protein metabolism.

[36] **Gregory, P. W., & Goss, H.** Growth **3**: 159. 1939.

MEDIATION OF GROWTH-PROMOTING EFFECTS BY OTHER ENDOCRINE GLANDS

A question that always arises in considering the alterations in function produced by the injection of a pituitary extract is whether the effects in whole or in part are a result of the direct action of some constituent of the extract or whether they are a consequence of the stimulation of some other endocrine gland for which the extract employed contains a specific "tropic" agent.

In the case of such a complex phenomenon as growth it may be assumed that there are certain conditions that must be observed before this can occur. Among these are an adequate supply of foodstuffs, together with the necessary vitamins and minerals, and in addition at certain stages of development not only an adequate quantity of hormones but probably the presence of these in definite proportions to each other. It is well known that growth may be retarded or even cease altogether if certain types of foodstuffs, minerals or vitamins are lacking in the diet. It is also known that a cessation of growth may be caused not only by the removal of the pituitary but also the removal of the thyroid, adrenals or pancreas. The reason for the cessation of growth may be very different in these various instances but nevertheless it becomes necessary to examine carefully the circumstances under which a pituitary extract may influence growth and to decide if possible whether the growth-promoting action is due to some specific effect of a separate hormone secreted by the gland that acts independently of all other members of the endocrine system or whether the stimulation of growth is not in part due to the simultaneous stimulation of certain other endocrine glands. If this were the case it might well be that the liberation of several hormones from the anterior pituitary in a certain proportion to each other could be said to constitute the effective growth stimulus. The evidence that has been advanced concerning the role of other endocrine glands is summarized under the individual organs considered.

THE THYROID

Removal of the thyroid causes a virtual cessation of growth that is particularly marked if the operation is carried out at an early age. Furthermore, the injection of an excessive quantity of the thyroid hormone will also bring about a retardation or cessation of growth.

Flower and Evans[37] found that young female rats dwarfed by thyroidectomy could be made to resume growth if given A.P.E. Similar

[37] Flower, C. F., & Evans, H. M. Anat. Rec. 29: 383. 1924.

results were found with adult thyroidectomized female rats. Smith and his collaborators[38] found that thyroid extract would not induce growth in hypophysectomized rats but the animals responded to A.P.E.

Later, Smith[39] reported that if both the thyroid and hypophysis were removed, better growth was obtained with A.P.E. if thyroid extract was simultaneously administered. Evans, Simpson and Pencharz[40] have repeated the earlier work of Flower and Evans and though confirming their observations have also found that the response to A.P.E. was greater if the animals were also given thyroid hormone. In their experiments also thyroxin failed to promote the growth of thyroidectomized-hypophysectomized animals.

It should be noted that in all these experiments thyroidectomy was performed sometime after birth and evidently this is a point of importance because Salmon[41] reported that if the rats are thyroidectomized at birth there follows not only a remarkable stunting of the animals but also an absolute failure to respond with a resumption of growth to the implantation of pituitary tissue or the injection of A.P.E. She suggested that the thyroid hormone is responsible for the development of the capacity of the organism to respond to other hormones.

Added interest has been given to these studies on the relation of the thyroid to the growth-stimulating properties of the pituitary by the work of Riddle and his collaborators. This work may be said to have begun by the observation of Bates, Laanes and Riddle[42] that the administration of prolactin, desiccated thyroid, or thyrotropic hormone to dwarf mice promotes growth and that there exists a synergism between prolactin and thyrotropic hormone which is sufficient to account for the growth-promoting effects of A.P.E. At that time these authors expressed the opinion that the "growth hormone" as a separate entity did not exist. Further work along these lines by this group of investigators has been largely confined to studies of the influence of various pituitary preparations on the growth and metabolism of hypophysectomized pigeons[43] and though their results suggest that prolactin may be of especial significance in its ability to maintain the appetite and promote both body growth and that of the intestinal organs in this species there is little to suggest that it has any such effect in a mammal. Indeed, Evans[44] in a spirited reply to their claims regarding the existence of a separate growth principle has pointed out that: (a) anterior pituitary extracts practically free of pro-

38 **Smith, P. E., Greenwood, C. F., & Foster, G. L.** Am. Jour. Path. **3**: 669. 1927.
39 **Smith, P. E.** Proc. Soc. Exp. Biol. & Med. **30**: 1252. 1933.
40 **Evans, H. M., Simpson, M. E., & Pencharz, R. I.** Endocrinology **25**: 175. 1939.
41 **Salmon, T. N.** Endocrinology, **23**: 446. 1938; **29**: 291. 1941.
42 **Bates, R. W., Laanes, T., & Riddle, O.** Proc. Soc. Exp. Biol. & Med. **33**: 446. 1935–36.
43 **Schooley, J. P., Riddle, O., & Bates, R. W.** Am. Jour. Anat. **69**: 123. 1941.
44 **Evans, H. M.** Proc. Assn. Res. Nerv. Mental Dis. **18**: 175. 1936.

lactin and thyrotropic hormone still produce an acceleration of growth in both normal and hypophysectomized rats; and by the injection of purified prolactin either alone or in combination with a potent thyrotropic extract failed to do so. White, Catchpole and Long[45] have also reported that their highly purified crystalline preparation of prolactin in doses of 4 mg. a day did not induce growth in hypophysectomized rats.

Apart from those experiments in which an increase in body weight has been taken as a measure of growth-promoting activity it has been shown by Gaebler[46] that thyroidectomy does not prevent the nitrogen-retaining effect of A.P.E. in the dog.

It may be concluded that, unless thyroidectomy is performed very early in life, the absence of this endocrine gland does not prevent either the ability of anterior pituitary extracts to induce growth nor their capacity to bring about nitrogen retention. Furthermore, the thyroid hormone is unable to induce growth in hypophysectomized animals.

The Adrenal Cortex

Though the evidence concerning the relation of the thyroid to the growth-promoting action of pituitary extracts is in general in agreement, the role of the adrenal cortex has not as yet been thoroughly investigated. The reasons for this are several and include not only the severe and rapidly fatal deficiency that occurs following removal of the adrenals but also the fact that adequate replacement therapy for this deficiency is a recent development. Certain observations have been made, however, that suggest that although the growth response to A.P.E. may be restricted in animals deprived of their adrenals nevertheless it is still evident. The administration of such potent adrenal steroids as corticosterone or 11-dehydro-17-hydroxy corticosterone does not cause growth in hypophysectomized rats,[47] although it is still an open question as to whether the use of such steroids along with purified growth extracts would not result in better growth than if the pituitary extract alone were given.[48] Indeed, as mentioned above, the superiority of crude extracts over long periods of injection may well be due to their added complement of adrenotropic (and thyrotropic) hormone.

The enhancement of growth in adrenalectomized animals by pituitary implants has been studied by Emery and Gottsch.[49] They reported that female adrenalectomized rats that received pituitary implants from

[45] **White, A., Catchpole, H. R., & Long, C. N. H.** Science **86**: 82. 1937.
[46] **Gaebler, O. H.** Am. Jour. Physiol. **110**: 584. 1935.
[47] **White, A., & Long, C. N. H.** Unpublished data.
[48] It should be pointed out that the administration of these steroids or of cortical extracts greatly increases the capacity of hypophysectomized rats to resist such stresses as fasting, insulin injection or exposure to cold.
[49] **Emery, F. E., & Gottsch, L. G.** Endocrinology **28**: 321. 1941.

castrated rats grew slightly faster than did their normal controls and far better than untreated adrenalectomized rats. Although the primary purpose of the investigation was to study the influence of *corpora lutea* formation on the survival of adrenalectomized rats this observation is of significance in indicating that pituitary implants are capable of producing better than normal growth in rats deprived of their adrenal glands. Furthermore, it should also be noted that the substitution of a potent gonadotropic extract, though effective in prolonging life, did not cause significant gains in weight.

In earlier experiments with alkaline extracts Evans and his associates[9] noted that although these preparations did not prolong the life of adrenalectomized rats they did in some instances produce a gain in weight. But this gain in weight was observed in only one group of rats that survived the operation for 20 days or more while those that lived less than this time always lost weight. Shumacker and Firor[50] have also found that pituitary implants do not influence the loss of weight or survival of adrenalectomized rats.

In our laboratory, Miss Fry and I have frequently administered growth-promoting extracts to adrenalectomized and partially depancreatized rats. In some animals no supportive treatment except the administration of 1 per cent NaCl solution as drinking fluid was given. In others the animals received a constant daily dose of adrenal cortical extract that was added to the drinking water. In general, it may be stated that even when the rats received only sodium chloride the injection of either crude or partially purified growth-promoting extracts nearly always was followed by a gain in weight. When the rats received a constant daily dose of cortical extracts, excellent gains in weight were always observed as shown in FIGURES 2 and 3. Our experience suggests that although A.P.E. is effective in causing a gain in weight in the absence of any cortical hormone nevertheless better responses always followed treatment with it. This, of course, might have been expected but the real point of these experiments is that, although the presence of some cortical hormone is necessary for a maximal response, such effects are not dependent on the presence of cortical cells.

However equivocal the influence of A.P.E. on the growth of adrenalectomized rats may appear there is another line of evidence that suggests (provided the severe results of adrenal insufficiency are avoided) that the protein metabolism of such animals responds in the same manner, if not the same degree, as normal animals to the injections of extracts that produce growth in the latter. Harrison and Long[29] injected

[50] **Shumacker, H. B., Jr., & Firor, W. M.** Endocrinology **18**: 676. 1934.

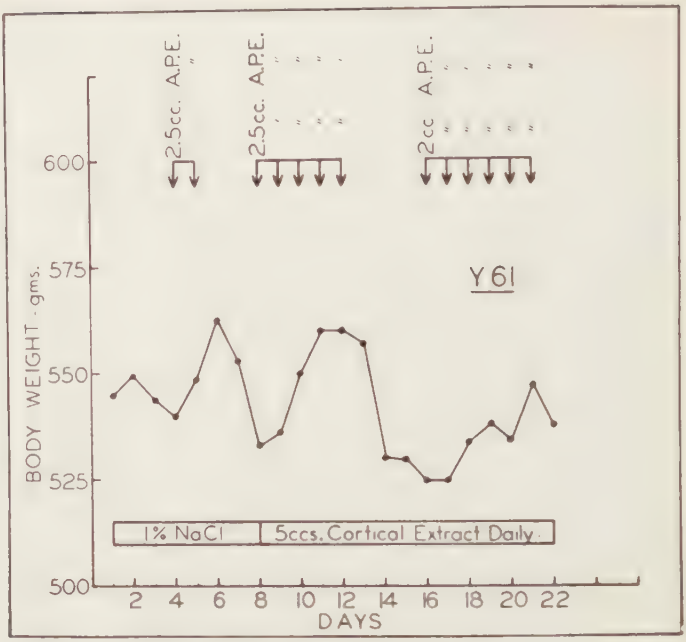

FIGURE 2.

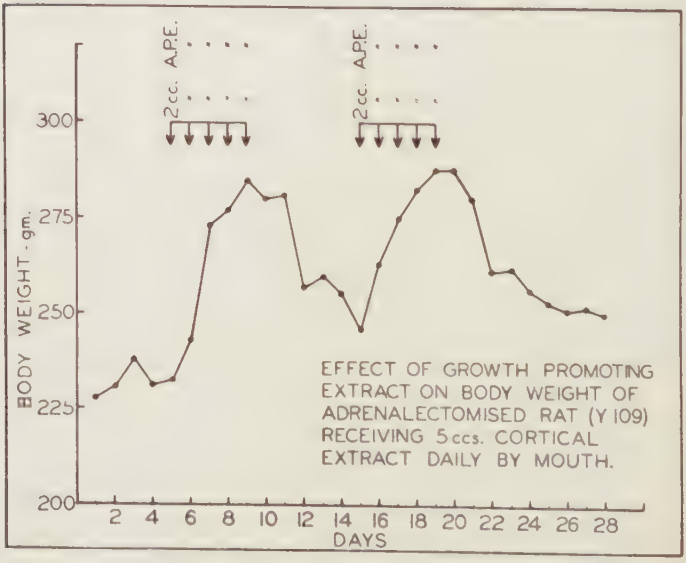

FIGURE 3. Effect of growth-promoting extract on body weight of adrenalectomised rat (Y 109) receiving 5 cc. cortical extract daily by mouth.

fasted adrenalectomized rats with A.P.E. and observed not only a fall in urine nitrogen of the same magnitude as found in intact animals but also a decrease in the blood N.P.N. It was also observed that the blood glucose level was decreased, particularly in the adrenalectomized rats, and that acetonuria was present after the injections.

Dr. Gaebler has permitted me to cite an experiment he has recently carried out on a dog with thyroid, pancreas and both adrenals removed. The diet was kept constant and the animal received daily injections of insulin and cortical extract. When 100–200 mg. of a potent growth-promoting preparation were injected in one dose there followed a moderate degree of nitrogen retention which, although less in magnitude than that previously found by him in normal animals, was nevertheless quite apparent. The weight of the animal also increased slightly after the injection.

The Pancreas

It has long been recognized that the injection of insulin decreases the nitrogen excretion in the urine provided ample carbohydrate is supplied in the diet. It is also common knowledge that an absent or deficient supply of insulin causes cessation or retardation of growth.

The particular interest in the role of the pancreas on the growth-promoting action of the anterior pituitary lies in the fact that the injection of crude extracts is followed by evidence of an impaired carbohydrate tolerance in most species and in some, particularly the dog, by glycosuria and ultimately a permanent diabetic state.

Since the growth-promoting and "diabetogenic" action are always closely associated in anterior pituitary extracts it is a point of considerable interest to know whether or not these two effects are different manifestations of the same hormone. This point will be considered in more detail in a later section; for the moment the question as to whether the presence of an intact pancreas is necessary for the growth-promoting action of A.P.E. may now be considered.

Mirsky[51] has suggested that the nitrogen-retaining effect of A.P.E. is due to stimulation of the islands of Langerhans by a "pancreatotropic" hormone and that the increased insulin supply not only decreases deamination in the liver but also increases nitrogen retention (protein synthesis) in the muscles. If this view is correct a depancreatized animal should show a notable failure to retain nitrogen when A.P.E. is injected. Mirsky believes he has demonstrated such a failure by his observations that A.P.E. increases the rate of accumulation of non-protein nitrogen

[51] **Mirsky, I. A.** Endocrinology **25**: 52. 1939.

in the blood of nephrectomized-depancreatized dogs, but the same extract given to nephrectomized but otherwise normal dogs results in a decreased rate of accumulation of these intermediary metabolites. He also supports this hypothesis by his observation that the injection of insulin also decreases the accumulation of the blood N.P.N. in normal, eviscerated and depancreatized dogs.[52]

Gaebler and Galbraith,[53] however, have carried out carefully controlled balance experiments on depancreatized dogs receiving a standard quantity of insulin and then injected with A.P.E. and conclude that an increased insulin output is not the immediate and only cause of the observed nitrogen retention although they concede that the presence of some insulin may be an essential condition for it. Finally, as mentioned above, Gaebler has shown that even if the thyroid and adrenals are removed in addition to the pancreas, nitrogen retention can still be observed after A.P.E. injection.

Since the above was written Young[54] has shown that when puppies in contrast to adult dogs are treated with A.P.E. they do not develop glycosuria, at least not until the injections have been carried on for some months; but they do respond by a marked gain in weight and nitrogen retention. Young suggests that these changes are associated with an increase in the quantity of islet tissue which can be regarded as then secreting sufficient insulin to neutralize at least for a time the "diabetogenic" activity of the extract.

In view of this and other work from his laboratory, Young suggests that the A.P.E. contains at least two principles that influence metabolism: (1) a pancreatotropic hormone which increases insulin secretion which in turn increases nitrogen retention and causes a gain in body weight; and (2) a diabetogenic hormone which either suppresses carbohydrate oxidation or increases carbohydrate formation.

Such a view might be interpreted to indicate that the pancreatotropic hormone through its influence on insulin secretion was a growth-promoting hormone, although it is hardly likely that the failure of hypophysectomized animals to grow is conditioned by an inadequate supply of insulin. It seems more reasonable to conclude that continuous growth requires a proportionately larger supply of insulin (and other hormones) and that so long as this can be maintained the growth hormone of the anterior pituitary will continue to operate in a normal manner. If the secretion of insulin becomes inadequate to meet the metabolic demands

[52] **Mirsky, I. A.** Am. Jour. Physiol. **124**: 569. 1938.
[53] **Gaebler, O. H., & Galbraith, H. W.** Endocrinology **28**: 171. 1941.
[54] **Young, F. G.** Brit. Med. Jour. **2**: 897. 1941.

imposed by rapid growth, the usual consequences of such a defect become manifest.

The Gonads

Although removal of the glands may influence the growth rate to some extent there is ample evidence that it does not modify in any significant manner the growth response to the injection of A.P.E. It should be remembered, however, that Kochakian and Murlin[55] and Kenyon and coworkers[56] have shown that the injection of testosterone propionate is followed by striking nitrogen retention and a gain in weight in dogs and man, respectively. In the experiments on human beings it could be calculated that a considerable part of this gain in weight was due to the deposit of newly formed protein in tissues other than those of the genital organs. Though it is improbable that such effects could be demonstrated in hypophysectomized animals nevertheless these experiments emphasize the complexity of the endocrine relationships controlling growth.

The Thymus

Although Bomskov and Sladovic[57] claimed that the diabetogenic effects of the anterior pituitary are mediated by the thymus, Reinhardt, Marx and Evans[58] have been able to show that at least as far as the growth-promoting action of A.P.E. in rats in concerned, removal of the thymus is without effect. It may be noted, however, that in a recent paper Marx and coworkers[13] found that the thymus gland of hypophysectomized rats treated with a purified growth preparation grows to a greater degree than that of any other organ examined.

It can be concluded that, although the absence of such essential hormones as these of the thyroid, adrenal cortex, or pancreas may obliterate or greatly modify the usual growth-promoting or nitrogen-retaining properties of anterior pituitary extract, there is little evidence to suggest that either of these effects is mediated by these endocrine glands and consequently it would appear that there exists in the anterior pituitary a specific agent that directly affects the tissues and whose action results in an increased retention of protein and an acceleration of the growth rate.

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[55] **Kochakian, C. D., & Murlin, J. R.** Jour. Nutrit. **10**: 437. 1935; Am. Jour. Physiol. **117**: 642. 1936; Endocrinology **21**: 750. 1937.
[56] **Kenyon, A. T., Knowlton, K., Sandiford, I., Koch, F. C., & Lotwin, G.** Endocrinology **26**: 26. 1940.
[57] **Bomskov, C., & Sladovic, L.** Pfluger's Arch. ges. Physiol. **243**: 611. 1940.
[58] **Reinhardt, W. O., Marx, W., & Evans, H. M.** Proc. Soc. Exp. Biol. & Med. **46**: 411. 1941.

IS THE GROWTH HORMONE A REGULATOR OF PROTEIN METABOLISM?

It has been stressed that not only is true growth characterized by an increased rate of protein synthesis but also that the material laid down in the gain in weight has a rather constant composition. It has also been noted that many other procedures besides hypophysectomy may inhibit growth, but the specific effect of the anterior pituitary appears to be its ability either to accelerate the growth rate of a growing animal or to cause a striking resumption of growth in those species in which it has practically ceased and in which certain prerequisites such as open epiphyses are still present. If a resumption of skeletal growth is not possible because of epiphyseal closure, it has been shown that this agent will cause a resumption of growth in the soft tissues of the body.

In approaching the question as to whether the growth hormone produces these effects solely by virtue of its influence on certain phases of protein metabolism, it is well to recall:

A. The fall in the urea and amino acid nitrogen content of the blood and liver begins soon after the injection of A.P.E. and persists throughout its period of action. This is also true of the decrease in the glutathione content of the liver and muscles observed by Gregory and Goss.

B. Nitrogen retention and a decrease in the non-protein nitrogenous constituents of the blood can still be produced in fasting or phloridzinized animals. Under such circumstances growth is of course not possible; indeed the animals are losing weight.

C. Most of the weight gained by animals after short periods of treatment with A.P.E. is lost when treatment is stopped, but if treatment is continued progressively smaller parts of the weight increment are lost when A.P.E. is finally withdrawn.[59] This strongly suggests that under the action of this hormone protein or its derivatives are first accumulated in a highly labile form, but as time goes on more and more of the retained protein is converted into forms which are not so easily yielded again.

D. The important role of the liver in protein metabolism has naturally directed attention to this organ as a possible site of the first action of the hormone.[60] It has been observed that the liver increases in size out of proportion to the rest of the body in animals treated with crude growth-promoting extracts.[60] Furthermore, the immediate changes

[59] **Lee, M. O.** Proc. Assn. Res. Nerv. Mental Dis. **18**: 193. 1936.

[60] Doubt on the relation of the growth hormone to the increase in liver size is raised by a recent paper by Fraenkel-Conrat, Simpson, & Evans.[61] These investigators found that purified growth hormone causes only a slight absolute increase and a significant relative decrease in the liver weight of hypophysectomized rats, whereas purified thyrotropic hormone or thyroxin bring about both a relative and absolute increase in liver weight.

[61] **Fraenkel-Conrat, H. L., Simpson, M. E., & Evans, H. M.** Am. Jour. Physiol. **135**: 398. 1942.

in such nitrogenous constituents of tissues as amino acids, urea and glutathione are far more striking in the liver than the rest of the body. It is also known, particularly from the work of Addis, Poo and Lew[62] that in a 2-day fast rats lose 20 per cent of their protein but only 4 per cent from the rest of the body. We have frequently observed that rats when hypophysectomized and then immediately fasted for 2 days excrete as much as 50 per cent more nitrogen than intact rats.

E. The work of Mirsky[61] indicates that nitrogen retention does not follow A.P.E. injection into eviscerated (liverless) animals and some preliminary experiments by Miss Frame in this laboratory also suggest that the usual decrease in blood amino acids is not elicited by A.P.E. in eviscerated rats

Apart from the question as to whether the sole primary site of action of the growth hormone is in the liver, a fairly reasonable hypothesis can be advanced that the participation of this agent in the growth process is due to its influence on protein metabolism. The nature of this effect is quite unknown but in general terms it is expressed either by an increased rate of formation of proteins or their immediate derivatives or by an inhibition of the rate of protein catabolism.

There are certain other implications of this statement that are of interest. The hypophysectomized animal not only has a disordered metabolism that is a consequence of the absence of the growth hormone but also exhibits the characteristic changes that follow hypofunction of other endocrine glands. The injection of a growth preparation freed from the tropic hormones may be expected to cause nitrogen retention and initiate growth but it is still a very open question as to whether such growth could be long continued if such organs as the adrenal cortex and thyroid were unable to increase their output of hormone to meet the increased demands placed on the organism by a continued increase in size. Questions such as this lead one to wonder whether the use of the term "growth" hormone for this pituitary agent does not place too great an emphasis on this particular agent as the only hormonal factor that is necessary for growth to occur. There is no doubt of its importance and probably little doubt that it is a separate entity but nevertheless as our knowledge of its sphere of influence is more fully defined it seems entirely probable that some other designation will ultimately be assigned to it than the one used at the present time.

When most animals reach a certain age growth practically ceases. Is it to be assumed that under these conditions the growth-promoting activity of the anterior pituitary is in abeyance? There is good evidence

[62] **Addis, T., Poo, L. J., & Lew, W.** Jour. Biol. Chem. **115**: 117. 1936.

that the growth hormone content of the gland is not greatly different in adult animals from that found in young animals at their most rapid period of growth. The answer to this may lie in the fact that protein metabolism is never in a stationary state and that the difference between young and adult animals is that in young animals protein synthesis is greater than protein breakdown, but in adult animals a fairly exact balance has been struck between the two. The work of Schoenheimer and Rittenberg[63] has clearly indicated that a constant exchange is going on between the nitrogen derived from the diet and that of the tissues and that, far from being the "building blocks" of living cells, the protein molecules are continually undergoing transformation. Furthermore, the rather exact balance in protein metabolism maintained in adult animals suggests the existence of factors capable of regulating both the anabolic and catabolic phases.

The evidence outlined above indicates that the growth hormone of the anterior pituitary is one of these regulatory factors and that its operation displaces the metabolism so that protein retention occurs. Such a displacement operates during the period of growth but in later life either its influence is less intense or other factors that accelerate protein catabolism come into greater prominence. Indeed, there is already some evidence that the secretions of the adrenal cortex influence protein metabolism in an opposite manner to that of the pituitary growth factor. Since, however, the functional activity of the adrenal cortex itself is controlled by the anterior pituitary this organ can adjust the protein metabolism within a wide range.

In spite of these generalizations it must be frankly admitted that little is really known of the mechanism of action of the growth hormone and to couple it with the problem of protein synthesis merely transfers the question to another field in which only fragmentary knowledge is available. Nevertheless, the continued study of this unique hormone may ultimately prove to be exceedingly valuable in this most difficult field of biochemistry.

In conclusion, it should be noted that Paschkis[64] and Paschkis and Schwoner[65] suggest that the factor which produces a decrease in the non-protein nitrogen of the blood is not identical with that which promotes growth. The main point of differentiation appears to be that gelatine feeding still produces a fall in the N.P.N. content of the blood of pituitary dwarfs who, of course, do not grow. In my opinion stronger evidence than this will be required to establish the separate identity of the

[63] **Schoenheimer, R., & Rittenberg, D.** Physiol. Rev. **20**: 218. 1940.
[64] **Paschkis, K. E.** Endocrinology **23**: 368. 1938.
[65] **Paschkis, K. E., & Schwoner, A.** Endocrinology **26**: 117. 1940.

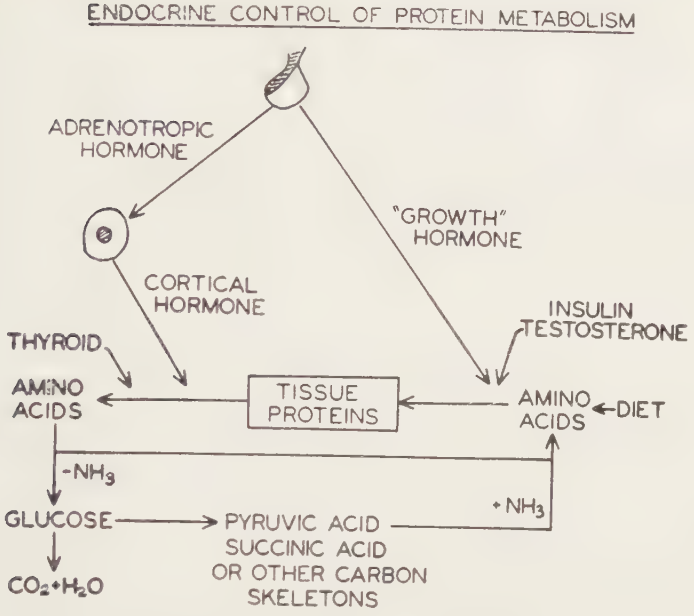

FIGURE 4. Endocrine control of protein metabolism.

two hormones. In FIGURE 4 is shown the endocrine factors that appear to regulate protein metabolism and their possible points of action.

ASSAY OF THE GROWTH HORMONE

The two universally used methods for the assay of the growth hormone are (a) the gain in weight produced in plateaued female rats and (b) the resumption of growth and consequent gain in weight of hypophysectomized rats. Both methods are laborious and time-consuming, particularly the latter, but at present they are the only methods that permit a reasonable degree of accuracy.

Evans and his colleagues[66] have undoubtedly had the most extensive experience in the assay of growth-promoting extracts. In their work they have used plateaued female rats in groups of 6 or more animals and judged the response by the average weight gained over a period of 20 days with 17 injections. The unit of activity was defined as the amount of material producing an average gain of 40 gm. in body weight per rat in this period. Their data show a linear response between growth and dose over a twentyfold increase in the material administered. Other

[66] **Marx, W., Simpson, M. E., & Evans, H. M.** Endocrinology **30**: 1. 1942.

investigators have used shorter periods of injection, some as short as 3 days, but as Evans points out the slope of the dose-response curve increases up to 15 days and consequently a longer period insures greater accuracy. Also in our laboratory we have frequently observed that temporary gains in weight (water retention ?) are not uncommon after the injection of the cruder extracts which probably contain appreciable amounts of posterior lobe hormones and possibly others such as the adrenotropic hormone which may also influence the electrolyte and water balance.

Many investigators have preferred to use the hypophysectomized rat as a test object. To supply and maintain sufficient numbers of these animals is no easy matter, although for "spot" tests of growth-promoting activity they can be advantageously used since a weight increase can usually be detected after only a few injections of an active preparation. They are also probably 10 times as sensitive as intact animals. Van Dyke and Wallen-Lawrence[67] have devised a method of assay in which only 3 daily injections are given. Although this method does not yield good quantitative results, it is undoubtedly useful for following the growth-promoting activity when fractionation of pituitary extracts is being carried out.

The majority of those who have used hypophysectomized rats for assay purposes have carried the injections over longer periods (7–20 days) and most workers have defined their unit of activity as the quantity of material that will produce an average gain of 1 gm. a day for the period chosen. It goes without saying that in carrying out these tests the ordinary precautions regarding biological assay must be observed. Among these are: the use of animals of a uniform age and strain who have been raised under identical conditions of diet and environment; the use of a sufficient number of animals at each dose level tested and the use of doses of the active principle that are submaximal in their effects; finally, although this is perhaps the most difficult to meet at the present time, in the case of the growth hormone the simultaneous injection of comparable groups of animals with a standard preparation of the material under test.

Observing such precautions Bülbring[68] has been able to show that for an injection period of 7 days there is a linear relationship between the gain in weight and the logarithm of the dose when 6 hypophysectomized rats are used in each group. She further notes that the response is not so uniform if the animals are used more than once.

[67] Van Dyke, H. B., & Wallen-Lawrence, Z. Jour. Pharmacol. & Exp. Therap. **40**: 413. 1930.
[68] Bülbring, E. Quart. J. Pharm. & Pharmacol. **11**: 26. 1938.

In the past there has been some discussion regarding the relative merits of the plateaued female rat and the hypophysectomized rat as test objects for growth-promoting activity[69] and, although there is much to be said in favor of the latter, the recent studies of Evans and his colleagues have shown that normal rats can be used as successfully as hypophysectomized ones. They also have the added advantage of possessing an intact endocrine system, a fact which as Evans states may be of importance as the purification of the growth-promoting activity is achieved.

Freud, Levie and Kroon[22] and Freud and Levie[70] have advocated a different method of assay that is based on their observation that growth of the tail vertebrae ceases after hypophysectomy but can be made to resume by injecting growth hormone. They indicate that treatment must be started soon after operation, otherwise the epiphyses will be closed and no response can then be obtained. They advocate the measurement of the tail length, preferably in skiagrams, as an index of growth promotion. Probably owing to the war no extensive quantitative data have been available for study, but Evans points out that in the experience of his laboratory tail growth continues for some time after hypophysectomy in young rats and that in any case measurement of tail length is no more accurate, if as accurate, as measurement of body weight. The further claim of Freud and his collaborators that the specific effect of growth hormone is a chondrotropic one is commented on elsewhere.

Attempts have been made to use certain alterations in the constituents of blood and tissues as a measure of growth-promoting activity; among these is the decrease in glutathione content of the liver after A.P.E. injection. Lee[59] commented that he found this method rather impractical and also stated that similar results followed an attempt to use the decrease of urea, amino acid, nitrogen, etc., in the tissues. But if it is ultimately shown that the same agent is responsible both for growth stimulation and the alterations in nitrogen metabolism, it would seem worthwhile to investigate more thoroughly the question as to whether some phase of protein metabolism might not be adopted as an assay method since there would be a considerable saving of time by the use of metabolic changes extending over a period of hours instead of days.

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[69] **Chou, C., Chang, C., Chen, G., & Van Dyke, H. B.** Endocrinology **22**: 322. 1938.
[70] **Freud, J., & Levie, J.** Arch. internat. pharmacodyn. et therap. **59**: 232. 1938.

PURIFICATION OF THE GROWTH HORMONE

The problem of the isolation of the growth hormone of the anterior pituitary is of interest not only because the preparation of the hormone in such a form would permit its chemical characteristics to be determined but also because it would then be possible to determine how many other metabolic activities were associated with the gland.

Although at the present time this desirable goal has yet to be achieved considerable progress has recently been made, so that now certain general statements can be made concerning its chemical characteristics. The substance is either a protein or a closely related protein derivative. It is destroyed by heat, inactivated by proteolytic enzymes and precipitated by the usual protein precipitants. It possesses a limited pH stability, being inactivated by strong acid solutions but is more stable in alkaline solutions. Consequently, the methods employed to extract the hormone from the gland have usually begun with the use of mildly alkaline or neutral solutions. Evans and his colleagues have employed $Ba(OH)_2$ or $Ca(OH)_2$ since these metals are easily removed. Others have used NaOH and Bonsnes and White[71] used 2 per cent NaCl solutions. Such initial extracts contain not only a major part of all the pituitary hormones but also considerable quantities of inert proteins. With such extracts a large part of the work on the phenomenon of growth promotion and other phases of anterior pituitary physiology has been done, but it is well to remember the essential crudity of composition of these extracts.

Further purification of the growth (and other principles) has followed the usual methods employed for the fractionation of protein mixtures: (a) precipitation with Na_2SO_4 or $(NH_4)_2SO_4$ and (b) isoelectric precipitation.

Thus Van Dyke and Wallen-Lawrence[67] adjusted the alkaline extract to pH 7.2 and added Na_2SO_4 to make the final concentration 20 per cent. The precipitate contained all the growth-promoting activity. The Na_2SO_4 precipitate was then dialyzed free from inorganic salt and the resulting solution adjusted to pH 4.8, the precipitate discarded and the supernatant was used both for animal and clinical studies. Bugbee, Simond and Grimes[72] also used the globulin fraction precipitated by Na_2SO_4 as their working solution. Extracts of this type, however, undoubtedly contain other pituitary hormones.

Evans and his colleagues have published a number of papers during the last 10 years on the purification of this hormone. In their first paper[9] there are extensive data on comparative methods of extraction and some

[71] **Bonsnes, R. W., & White, A.** Endocrinology **26**: 990. 1940.
[72] **Bugbee, E. P., Simond, A. E., & Grimes, H. M.** Endocrinology **15**: 41. 1931.

of the properties of the hormone. In 1938 they reported[73] the use of $(NH_4)_2SO_4$ as a precipitating agent for the hormone. The alkaline extract was progressively precipitated by increasing the $(NH_4)_2SO_4$ concentration in steps from $\frac{1}{5}$ to $\frac{1}{2}$ saturation. The lower concentration did not precipitate the growth activity but half saturation did. This precipitate was freed from a good deal of inert material by repeated re-solution and precipitation but the final 'L' precipitate still contained considerable quantities of lactogenic, thyrotropic and gonadotropic hormones in addition to the growth hormone. The relative distribution of the 4 hormones had, however, been considerably altered from that found in the original extract.

From this 'L' fraction considerable quantities of gonadotropic hormone could be removed by extraction with 5–10 per cent NaCl in which it is much more soluble than the growth hormone. To remove the larger part of the lactogenic hormone either extraction with 0.1 saturated $(NH_4)_2SO_4$, in which it is more soluble than the growth hormone, or treatment with bromine water was employed. The last procedure precipitates the lactogenic hormone, and the growth principle can be recovered by saturating the supernatant with NaCl. Efforts to remove all the thyrotropic hormone were not too successful but a few preparations were obtained that contained only small quantities of this activity.

The whole procedure gave about a 70 per cent yield of growth hormone but the increase in potency of only fivefold was somewhat disappointing since so much of the other hormones had been removed.

In their most recently published paper[74] these investigators have introduced treatment of the "globulin" fraction precipitated by $(NH_4)_2SO_4$ with cysteine as a method of removing other hormones. To employ this, concentrated protein solutions (5–7 per cent) are treated with alkaline cysteine solution at room temperature for 1–2 days. The precipitate that forms is removed and the growth hormone isolated from the supernatant. This preparation contained 15.7–16.3 per cent nitrogen and practically no glucosamine in contrast to the gonadotropic hormone. It had about twice the growth activity of the 'L' fraction but still possessed considerable adrenotropic activity. It is of particular interest that this preparation lowered the respiratory quotient of glucose-fed rats and also stimulated ketogenesis.

Both these procedures, though producing some increase in potency, appear to be directed more toward removal or inactivation of other hormones than actually effecting an isolation of the growth principle.

[73] **Evans, H. M., Uyei, N., Bartz, Q. R., & Simpson, M. E.** Endocrinology **22**: 483. 1938.
[74] **Fraenkel-Conrat, H. L., Meamber, D. L., Simpson, M. E., & Evans, H. M.** Endocrinology **27**: 605. 1940.

Collip, Selye and Thomson[75] extracted the glands with 1 per cent NaOH or NH_4OH. The mixture was acidified with acetic acid and filtered; the residue was re-extracted with alkali and the acidification was repeated. The combined supernatants were made alkaline with 1 per cent NH_4OH and sufficient quantities of calcium chloride and sodium phosphate were added to form a suspension of calcium phosphate. Ammonia was removed by vacuum distillation and the calcium phosphate was collected and extracted with 0.5 per cent NaOH. This alkaline extract was adjusted to pH 6.5, made alkaline with NH_4OH and concentrated *in vacuo*. A precipitate settled out at pH 7.5–8.0 which was removed and extracted with alkali. This solution was neutralized and proved to have marked growth-stimulating properties. Such extracts were free of gonadotropic and thyrotropic hormone but contained prolactin.

Dingemanse[76] and Dingemanse and Freud[77] have used somewhat different procedures to produce an active growth preparation which they claimed is free from lactogenic and thyrotropic hormones. The material is absorbed on "Norite" from alkaline extract of acetone-dried glands. The hormone was elicited from the "Norite" with liquid phenol. This solution was poured into alcohol-ether and the precipitate collected. Empirical analysis showed a typically protein composition. They stated that the hormone is destroyed by heat, strong acids and alkalis, and is inactivated by proteolytic enzymes. They also stated that the active principle is ultrafilterable through collodion membranes of 30μ porosity. No lactogenic, thyrotropic, gonadotropic or adrenotropic activity could be detected at 30γ levels which was about 3 times the quantity required to produce a gain of 1 gm. a day in hypophysectomized rats.

In the earlier paper[77] these investigators also stated that quite an active growth preparation can be obtained by simply dialyzing a weak alkaline extract through collodion. After treatment of the dialysate with "Norite," active preparation was obtained that contained only 4.84 per cent (?) of nitrogen.

It should be noted that the claims for such a high degree of potency of this preparation could not be substantiated by Evans[44] and that Schooley, Riddle and Bates[38] stated that a preparation obtained from the Amsterdam laboratory contained detectable quantities of prolactin and traces of thyrotropic and gonadotropic activity and that in a daily dose

[75] **Collip, J. B., Selye, H., & Thomson, D. L.** Proc. Soc. Exp. Biol. & Med. **30**: 544. 1933.
[76] **Dingemanse, E.** Kongressbericht des XVI Internationalen Physiologen Kongress. Frei Vereinigung Schweiz. Physiologen. Zurich. 1938. p. 320.
[77] **Dingemanse, E., & Freud, J.** Acta brev. Neerland. **5**: 109. 1935.

of 0.1 mg. it did not stimulate growth in hypophysectomized pigeons, which was a marked contrast to the effect of 0.067 mg. of prolactin.

Fevold and coworkers[78] have reported a method by which 5 anterior lobe hormones can be separated into 5 different protein fractions. When precipitation of a pH 8 extract was carried out at pH 5.4 in the presence of 0.25 M $(NH_4)_2SO_4$ the growth, lactogenic, thyrotropic and gonadotropic hormones remained in the solution. The growth hormone was then precipitated by addition of $(NH_4)_2SO_4$ to 1.8 M concentration. Teel[79] had previously reported precipitation of the growth activity by 1.4 M Na_2SO_4. The precipitate obtained, after removal of $(NH_4)_2SO_4$, was extracted with 0.25 M $(NH_4)_2SO_4$ at pH 7.0. The solution was adjusted to pH 5.4 and an inert precipitate removed, the solution being rapidly readjusted to pH 7.0. From this the globulins were slowly precipitated by allowing $(NH_4)_2SO_4$ to diffuse into the solution. This precipitate was again extracted with 0.25 M $(NH_4)_2SO_4$ and the procedure was repeated. Finally, a product was obtained that assayed 115 growth units per mg. which the authors stated represents a concentration of 160-fold.

White and Bonsnes[80] made use of the ultracentrifuge in preparing growth extracts. A 2 per cent NaCl extract of beef anterior lobes was adjusted to pH 5.5, the precipitate discarded, and the supernatant adjusted to pH 4.9. The resulting precipitate was dissolved, adjusted to pH 7.4 and ultracentrifuged for 2 hours at 750 r.p.s. (142,000 g.). The supernatant fluid was removed from any sedimented material and the pH adjusted to 4.9. The precipitate, although still containing recognizable quantities of other pituitary hormones, had a marked growth-promoting activity in hypophysectomized rats.

In TABLE 3 are listed some of the preparations outlined above, together with an estimate of the quantity of material or nitrogen required to produce the unit response in either normal plateaued female or hypophysectomized rats. It will be observed that several groups of investigators have obtained preparations in which approximately 10 to 20 γ of the solid material, containing 1 to 2 γ of nitrogen, when administered daily to hypophysectomized rats would produce a gain of 1 gm. a day. None of these preparations can be considered to represent an isolation of the growth hormone in pure form, since they are either contaminated with other active principles or else still contain inert protein material.

78 **Fevold, H. L., Lee, M. O., Hisaw, F. L., & Cohn, E. J.** Endocrinology **26**: 999. 1940.
79 **Teel, H. M.** Science **69**: 405. 1929.
80 **White, A., & Bonses, R. W.** Unpublished data.

TABLE 3
PREPARATIONS OF GROWTH HORMONE

Investigators	Type of preparation	N content gm. per cent	Assay: Plateaued-female rats: Solids mg./unit	Assay: Plateaued-female rats: Nitrogen γ/unit	Assay: Hypophysectomized rats: Solids mg./unit	Assay: Hypophysectomized rats: Nitrogen γ/unit
Evans & Simpson (1933)	1. Alkaline extract	13–18	6–17	1040–2300	—	—
	2. Acetone-dried powder	13–25	5–13	970–2300		
	3. Isoelectric precipitates from (2)	12–18	5	580–800	—	—
	4. Supernatant from (3)	8–17	5	350–790	—	—
	5. 20% Na_2SO_4 precipitate	14–15	5–9	700–1300	—	—
Evans, *et. al.*, (1940)	6. Alkaline extract	16 (?)	7.2	1150 (?)	—	—
	7. $Ca(OH)_2$ extract	16 (?)	0.8	128 (?)	—	—
	8. 'L' fraction $(NH_4)_2SO_4$ precipitate	16 (?)	1.3	208 (?)	0.05–0.10	7.5–15.0(?)
	9. Globulin fraction	16 (?)	0.8	128 (?)	0.012–0.024	1.9–3.8 (?)
	10. Cysteine-treated globulin fraction	16 (?)	0.5	80 (?)	0.012–0.040	1.9–6.4 (?)
Van Dyke & Wallen-Lawrence	11. 20% Na_2SO_4 precipitate and isoelectric precipitation	5 (dry weight)	—	—	0.35	17.5 per 100 gm. rat)
Dingemanse & Freud (1935)	12. Acetone powder	—	—	—	6–10	—
	13. Dialysate absorbed on "Norite"	14.3	—	—	0.01	1.4
Collip, *et al.*, (1933)	14. Adsorbate on $Ca_3(PO_4)_2$	16 (?)	—	—	0.10	16.0 (?)
Fevold, *et al.*, (1940)	15. pH 8.0 extract of glands	16 (?)	—	—	1.3	208 (?)
	16. pH 5.4 solution in 0.25 M $(NH_4)_2SO_4$	16 (?)	—	—	0.25	40 (?)
	17. Repeated $(NH_4)_2SO_4$ precipitation	16 (?)	—	—	0.009	1.4 (?)
White & Long (1941)	18. Isoelectric precipitation and ultracentrifugation	14.1	—	—	0.025	3.8

METABOLIC CHANGES INDUCED BY ANTERIOR PITUITARY EXTRACTS

A large number of alterations in many phases of metabolism have been reported to follow the injection into normal animals of anterior pituitary extracts of various types. Indeed, so many observations have been reported that it is not an easy matter to present any coherent explanation of the series of events that occur. These difficulties arise by reason of the fact that the anterior pituitary secretes not only hormones that influence the tissues directly but also those that exert their effect by stimulating other endocrine glands.

It may be stated that there are four possible ways in which an anterior pituitary extract may influence the metabolism of the organism:

A. By those hormones whose secretions act directly on the tissues; this effect may be one involving practically all the tissues or may be limited to certain organs. Examples of this type are probably the growth and lactogenic hormones.

B. By the stimulation of other endocrine glands through the specific tropic hormones contained in the extract. Examples of these are the thyrotropic and adrenotropic hormones. Since the effects that follow the action of tropic hormones are due to the particular hormones they liberate, it follows that for an understanding of this particular type of pituitary action we must have information regarding the mode of action of such glands as the thyroid and adrenal cortex. Although much information of this character is available it is by no means complete, so that often considerable doubt exists as to whether a particular effect is attributable to their action.

C. The third possibility is one that does not directly involve either of the two foregoing types of pituitary hormones. It is that the alterations in metabolism produced by an anterior pituitary extract may ultimately involve an alteration in function of another endocrine gland which itself is not directly under the control of the anterior pituitary. The best example of this kind is the behavior of the insulin-secreting cells of the pancreas.

D. Finally, to render the situation still more complicated, the long-continued injection of anterior pituitary extracts may induce a condition in which the animal will neutralize not only the active principles in the extract injected but apparently also the secretion of his own pituitary. Thus, Severinghaus and Thomson[81] have reported that the long-continued injection of such extracts into dogs leads to atrophy of the gonads

[81] **Severinghaus, A. E., & Thomson, K. W.** Am. Jour. Path. **15**: 391. 1939.

and thyroid, and the occurrence of characteristic changes in the animal's pituitary. Consequently, such animals, far from exhibiting the metabolic changes associated with hyperthyroidism, actually showed those associated with hypofunction of this gland.

In view of all these possibilities it is not surprising that what amounts to despair has colored much work in this field. Some investigators have chosen to assume that each newly observed metabolic aberration was due to the operation of a specific metabolic hormone so that it is quite easy to compile a long list of "metabolic" hormones which on my last count had reached a total of 9 and which if added to other known active principles places this organ in a class by itself, for although many steroids have been isolated from such endocrine glands as the adrenal cortex and gonads only a small number have biological activity.

It is my opinion that the number of "metabolic" hormones is much smaller than has been suggested, but before discussing this further it would be well to review briefly the major changes in metabolism that follow the injection of crude anterior pituitary extracts into normal animals.

(1) General metabolism
  (a) Increased basal metabolic rate.
  (b) Growth.

(2) Carbohydrate metabolism
  (a) In fasted animals A.P.E.decreases the blood glucose content.
  (b) In fed animals, particularly those fed carbohydrate, A.P.E. produces hyperglycemia, a depression of the respiratory quotient and an increased accumulation of muscle and liver glycogen (glycostatic action). In the dog the effects are much more pronounced. Glycosuria occurs and if the injections are long continued a permanently diabetic state is established (diabetogenic action).
  (c) A.P.E. produces an increased resistance to the hypoglycemic action of insulin (glycotropic action).
  (d) In the rat A.P.E. brings about an increased insulin content of the pancreas but in the dog there follows a rapid decrease in insulin content.

(3) Protein metabolism
  (a) In fed, fasted or phloridzinized animals a single injection of A.P.E. is followed by a decreased nitrogen excretion in the urine and a decrease in the non-protein nitrogen content of the blood.
  (b) With continued injections of A.P.E. animals exhibit a gain

in weight which is characterized by a greater proportion of water, ash and protein retention and a smaller proportion of fat than are found in pair-fed untreated animals.

(4) Fat metabolism

(a) In fasted animals A.P.E. causes an increased rate of fat catabolism as is shown by an increased content of acetone bodies in the blood and the occurrence of acetonuria (ketogenic action). This also occurs in fed dogs but is not so marked in other species.

(b) There is also an accumulation of liver fat and a decreased fat content of the carcass.

(5) Salt and water metabolism

(a) Diuresis.

(b) A retention of potassium, phosphorus and calcium elements associated with the growth of the tissues and skeleton.

In attempting to interpret these changes in terms of the various hormones and metabolic interrelationships that are involved it is an advantage to consider them in terms of the various ways suggested above in which the anterior pituitary hormones may influence metabolism.

METABOLIC EFFECTS ELICITED BY TROPIC HORMONES

The Thyroid

The increased basal metabolism that follows A.P.E. injection is due at least in part to thyroid stimulation. Certain reservations must be made even for this statement. In fed animals the increase in oxygen consumption occurs soon after the A.P.E. is injected and is not prevented by removal of the thyroid.[46,82,83] The slower increase, occupying several days, which is characteristic of the thyroid hormone itself is abolished by a thyroidectomy prior to the injection of A.P.E.

Hypophysectomized rats have a decreased intestinal absorption rate of glucose[84] and the same was shown to be true in thyroidectomized rats.[85] Russell[86] demonstrated that the defect in hypophysectomized rats was due to thyroid hypofunction by showing that treatment of these animals with thyroxin restored the absorption rate to normal and increased the basal metabolic rate without, however, correcting the other metabolic deficiencies of these animals. Single injections of A.P.E. into normal animals over short experimental periods does not alter the glucose ab-

[82] **O'Donovan, D. K., & Collip, J. B.** Endocrinology **23**: 718. 1938.
[83] **Riddle, O., Smith, G. C., Bates, R. W., Moran, C. S., & Lahr, E. L.** Endocrinology **20**: 1. 1936.
[84] **Phillips, R. A., & Robb, P.** Am. Jour. Physiol. **109**: 82. 1934.
[85] **Althausen, T. L., & Stockholm, M.** Am. Jour. Physiol. **123**: 577. 1938.
[86] **Russell, J. A.** Am. Jour. Physiol. **122**: 547. 1938.

sorption rate[86] since there is not time for the hyperactivity of the thyroid to be exerted. It is to be anticipated, however, that after longer periods of treatment an increased rate of absorption would be observed.

There are, of course, other effects of thyroid stimulation which will become more manifest the longer the time of treatment with A.P.E. For example, Fraenkel-Conrat, Simpson and Evans[87] have shown that a marked absolute and relative increase in liver size follows treatment of rats with purified thyrotropic hormone. It was also demonstrated that an increased liver size follows the injection of purified adrenotropic hormone but growth preparations freed of these two substances bring about a relative decrease in liver size. Since whole pituitary extracts cause an increase in liver size[7] this work furnishes a good example of the clarification of the problem that will follow the successful separation of the various pituitary factors.

The Adrenal Cortex

The role played by the adrenal cortex in the regulation of metabolism has remained very obscure until recent years. It is now known that this gland is not only a regulator of the salt and water balance of the body but also through some of its hormones, notably those of the corticosterone type, it exerts an influence on protein and carbohydrate metabolism. These last effects are as follows: (1) Injection into fasting animals increases to a marked degree the liver glycogen content and to a less degree the blood glucose level, the muscle glycogen remaining unchanged. (2) Coincident with the absolute increase in the carbohydrate content of the body there is an increase in urine nitrogen. This increased quantity of protein catabolism is sufficient in magnitude to account for the extra carbohydrate found.[87] In other words, gluconeogenesis from protein is stimulated by these hormones which in turn implies that under their influence the rate of protein catabolism is increased. This interpretation of the action of these hormones finds support in certain characteristics of the metabolism of adrenalectomized animals which in turn have much in common with those observed to follow hypophysectomy.

When fasted both hypophysectomized and adrenalectomized animals develop hypoglycemia, the former, however, more rapidly and to a greater degree than the latter. Both have a much reduced liver glycogen content but adrenalectomized animals maintain their muscle glycogen fairly well, but those of hypophysectomized animals are rapidly depleted. This last fact, together with the high rate of carbohydrate utilization of

[87] **Long, C. N. H., Katzin, B., & Fry, E. G.** Endocrinology **26**: 309, 1940.

the latter constitutes a very significant difference in the metabolism of the two types of endocrine deficiency and indicates that not all the defects in carbohydrate metabolism after hypophysectomy are to be attributed to adrenal cortical hypofunction.

Both preparations exhibit significant defects when placed under conditions which normally invoke an increased rate of protein catabolism and glyconeogenesis. Among these are exposure to cold, pyrogenic agents, pancreatic diabetes, phloridzin diabetes, exposure to low oxygen pressures and, finally, such a comparatively mild circumstance as fasting. In all these instances not only is the rate of protein catabolism, as measured by the nitrogen excretion, significantly lower than normal but also the rate of gluconeogenesis as measured by the carbohydrate levels of the body or glycosuria. Indeed, the profound hypoglycemia and depletion of carbohydrate stores that occur in fasting hypophysectomized rats may be prevented by the administration of adrenal cortical hormones.

One point of difference in the behavior of the protein metabolism in these two glandular deficiencies should, however, be noted. Immediately after hypophysectomy in rats there is a greatly increased rate of nitrogen excretion, but after adrenalectomy the output is much less than normal.[88] If the hypophysectomized rats are tested some time after the operation, however, an actual reduced nitrogen excretion may be observed, possibly due in part to the ensuing adrenal atrophy and in part to the depletion of, and inability to restore, any depot protein. These observations not only emphasize the complex character of the metabolism after hypophysectomy but also indicate the biphasic character of the endocrine control of the protein metabolism.

Another fact in common to hypophysectomized and adrenalectomized animals is their extreme sensitivity to insulin. Furthermore, the hypoglycemic action of insulin in normal animals can be greatly reduced or abolished by the injection of A.P.E. (glycotropic action). Jensen and Grattan[89] have shown that the injection of cortical extract or adrenal steroids of the corticosterone type will also abolish the convulsive action of insulin in mice. Finally, they were able to show that the same result could be obtained by treatment of the animals with a fairly well purified adrenotropic preparation. Consequently, they suggested that the "glycotropic" hormone of the anterior pituitary was identical with the adrenotropic hormone and that the protective action was due to the liberation of the adrenal cortical steroids. These increased the available carbohydrate of the body by increasing the liver glycogen and this

[88] **Fry, E. G., & Long, C. N. H.** Unpublished data.
[89] **Jensen, H., & Grattan, J. F.** Am. Jour. Physiol. **129**: 270. 1939–40.

in turn moderated the hypoglycemic action of injected insulin. It might be pointed out, however, than an anti-insulin action of A.P.E. could also be achieved by an agent that inhibited glucose utilization in the tissues, so there is the possibility that two types of "glycotropic" action are invoked by crude A.P.E., although, as will be shown, there is also evidence that certain adrenal steroids inhibit carbohydrate utilization in the peripheral tissues.

One of the most dramatic consequences of the injection of anterior pituitary extracts into fed animals, particularly dogs, is the development of hyperglycemia and glycosuria accompanied by other signs of the diabetic state such as the occurrence of acetonuria. In other species this "diabetogenic" action is less marked but nevertheless can be detected by such procedures as the measurement of the respiratory quotient or glucose tolerance curve; both of which indicate that under the influence of A.P.E. there is a marked depression of the ability of the organism to utilize carbohydrate. The counterpart of the "diabetogenic" action of A.P.E. is the alleviation of a total pancreatic diabetes by hypophysectomy — the Houssay phenomenon. Though it has been maintained that both the "diabetogenic" and Houssay effects were not mediated by other endocrine glands, Long and Lukens[90] were able to show that adrenalectomy also alleviated a total pancreatic diabetes to a comparable degree. In addition, Long, Katzin and Fry[87] found that the diabetes of partially depancreatized rats could be aggravated by adrenal cortical steroids of the corticosterone type. Finally, Ingle[91] has been able to produce profuse glycosuria in a normal rat injected daily with 11-dehydro-17-hydroxy corticosterone.

These experiments not only suggest that a large part of the "diabetogenic" activity of the anterior pituitary is mediated by the adrenal cortex, but that this endocrine gland is capable of stimulating gluconeogenesis in the liver and of inhibiting glucose utilization in the tissues. It might be noted that these two effects have long been regarded as the characteristic disturbances of diabetic metabolism.

ANTERIOR PITUITARY FACTORS ACTING DIRECTLY ON THE TISSUES

If we exclude the possibility that all of the "diabetogenic" action of A.P.E. is exerted directly on the tissues we must still consider what hormones of this gland are to be regarded as exerting their full effects in this manner. Of these the lactogenic and growth-promoting hormones are

[90] **Long, C. N. H., & Lukens, F. D. W.** Jour. Exp. Med. **63**: 465. 1936.
[91] **Ingle, D. J.** Endocrinology **29**: 649. 1942.

the only ones that are sufficiently well defined, although even in the case of these hormones it has been shown that the presence of certain other hormones is necessary for their complete action.

The fact that extracts containing the growth hormone reduce the quantity of protein undergoing catabolism implies that the organism must shift to some other foodstuff to replace the calories lost by this reduction in the proportion of protein in the metabolic mixture. One might anticipate, therefore, that the injection of growth-promoting extracts would increase fat catabolism which under suitable conditions would give rise to acetonemia and acetonuria as well as increasing the quantity of the liver fat. It will also be recalled that in the experiments of Lee and Schaffer the treated animals gained more protein and less fat than the controls, indicating again that the burden of supporting the metabolism had been shifted to fat. When to this effect of growth hormone is added the effects of an agent that can inhibit carbohydrate utilization it can well be realized that the readjustment in metabolism produced by A.P.E. greatly increases fat catabolism so that it may be suggested that the ketogenic action of such extracts is not directly related to any pituitary agent that controls fat metabolism exclusively, but is a result of changes induced by those agents that control other phases of metabolism.[92]

It may also be pointed out in connection with the experiments of Lee and Schaffer that the increased gain in protein by the treated rats was not isocalorically equivalent to their increased utilization of fat. The treated group of animals had an excess of 141 gm. of protein equivalent to 578 cal. while they had utilized 119 gm. more fat than the controls which is equivalent to 1095 cal. Whether this difference is due to an increase in the basal metabolic rate of the treated group or whether it it may be taken to imply that there is some more significant relationship between protein synthesis and fat utilization are matters for conjecture. It is evident, however, that such an increased quantity of protein synthesis must require a considerable expenditure of energy and this may well have to be met by the combustion of a substance that is not only available in large quantity but also has a high calorific value. Since we are ignorant of so many phases of protein synthesis the suggestion that fat utilization is intimately coupled with this process may be worth further study.

In fasting animals the injection of A.P.E. also reduced protein catabolism. Since during fasting the amino acids furnish the sole source of glucose, it is noteworthy that such injections produce not hyperglycemia

[92] **Campbell, J., & Keenan, H. C.** Am. Jour. Physiol. **131**: 27. 1940, state that the pituitary factor that increases the liver fat of mice is not identical with the ketogenic activity of the extract.

change (except lactation) has so far been observed to follow the injection of this hormone into mammals.

There are also reports which suggest that the anterior pituitary hormones are not the only ones that may cause alterations in the insulin content of the pancreas. Griffiths, Marks and Young[101] found that, following the implantation of tablets of diethyl stilbestrol, estriol and estradiol into rats, the insulin content of the pancreas was increased; cholestrol, estrone and testosterone were inactive. Funk and coworkers[102] also stated that stilbestrol and estradiol increased the insulin content and that progesterone and testosterone caused a small decrease. It should be noted that stilbestrol produced glycosuria in normal rats[103] though it is stated that the estrogens aggravated the glycosuria of partially depancreatized ferrets.[104] Fraenkel-Conrat and collaborators,[107] however, have recently reported that this effect of estrogens in increasing the insulin content is mediated by the anterior pituitary since they are ineffective in this regard in hypophysectomized rats.

The marked effects of certain adrenal steroids on carbohydrate metabolism is discussed elsewhere. Haist and Best[108] reported that adrenalectomy did not alter the insulin content of rats given a balanced diet and maintained with sodium chloride. The administration of a high fat diet led to a decrease in insulin content but when 3 to 6 cc. of cortical extract was given by mouth to adrenalectomized rats on a balanced diet no change could be detected in the insulin content of the pancreas. But since 11-dehydro 17-hydroxy corticosterone will produce glycosuria in normal rats,[91] it would be well to reserve judgment on the influence of adrenal steroids of this type since the quantities of extract given in these experiments may not have been sufficient to produce detectable effects.

Present evidence would indicate that the production of insulin is not directly dependant on endocrine factors. It seems entirely probable that the demands of the organism expressed through the level of the blood glucose are the factors that regulate insulin production and supply. Circumstances that depress the general level of blood glucose lessen the requirements for insulin but those that result in an elevation of blood glucose stimulate an increased insulin production. A continued stimulation such as is produced by a continued high level of blood glucose appears to bring about, at least in some species, an ultimate failure of the insulin secretory mechanism.

[101] **Griffiths, M., Marks, H. P., & Young, F. G.** Nature **147**: 359. 1941.
[102] **Ingle, D. J.** Endocrinology **29**: 838. 1941.
[103] **Dolin, G., Joseph, S. J., & Gaunt, R.** Endocrinology **28**: 840. 1941.
[107] **Fraenkel-Conrat, H. L., Herring, V. V., Simpson, M. E., & Evans, H. M.** Proc. Soc. Exp. Biol. & Med. **48**: 333. 1941.
[108] **Haist, R. E., & Best, C. H.** Am. Jour. Physiol. **133**: 310. 1941.

The apparent importance of the blood glucose level is illustrated by the experiments of Lukens and Dohan. These investigators have previously shown[109] that insulin treatment of cats with a permanent diabetes induced by anterior pituitary extract was followed by morphological and functional recovery of the pancreatic islands. They recently found[110] that a course of phloridzin injections, which markedly lowered the blood glucose of these animals, was also followed by recovery as judged by the subsequent absence of glycosuria and a normal blood glucose level.

The experiments quoted above furnish an excellent example of the manner in which hormones that are concerned with one particular effect on metabolism—in this case the repression of glucose utilization and the stimulation of glucose production—may so alter the character of the metabolism that other endocrine glands may be involved, first by increasing their hormone production and finally, if the initial stimulus is long continued, by an actual decline in function. If the original stimulus is withdrawn, the abnormality in metabolism will be perpetuated by the deficiency in the endocrine that was secondarily involved.

Three ways have been outlined in which the anterior pituitary gland may influence the processes of metabolism and an attempt has been made to present examples of each kind of effect that may be produced as well as the metabolic interrelationships that may be involved. The fourth possibility has not been mentioned, namely, the "anti-hormone" effects that may be produced by long continued injection of A.P.E., since it seems that this can hardly be a physiological process and also it is extremely unlikely that the hormones in a pure form will produce "anti-hormones."

Enough, however, has been said to illustrate the complicated character of the endocrine control of metabolism, but in spite of the fact that emphasis has been placed on the influence of the hormones on metabolism, it should not be forgotten that the ability to utilize or oxidize foodstuffs is an inherent property of the cells and that the endocrine glands only accelerate or inhibit certain phases of these transformations.

Thus, although it has been said in the past that in the absence of insulin the organism is unable to utilize glucose, recent research has shown that the removal of the hypophysis or adrenals restores to some degree the ability to oxidize this carbohydrate. Similarly, thyroidectomy, although reducing the rate of oxidation in cells, does not completely suppress it and hypophysectomy, although greatly retarding protein synthesis,

[109] **Lukens, F. D. W., & Dohan, F. C.** Science **92**: 222. 1940.
[110] **Lukens, F. D. W., & Dohan, F. C.** Am. Jour. Physiol. **133**: 368. 1941.

does not entirely prevent growth since regeneration of such organs as the liver, although somewhat retarded, still occurs after its removal.

The exact manner in which a hormone diverts the metabolism in one direction or another is still unknown. It may be that just as certain vitamins have been shown to be parts of enzyme systems concerned with particular chemical transformations, so ultimately a similar role will be assigned to the hormones.